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(54) Title: MODIFIED OLIGONUCLEOTIDES FOR USE IN RNA INTERFERENCE

(57) Abstract: The present invention provides modified oligonucleotides for use in the RNA interference pathway of gene modulation. At least one nucleoside has a 2'-modification other than hydroxyl that gives an RNA like 3'-endo sugar conformation. The modified oligonucleotides are also provided having a 5'-phosphate group.

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## 5     **MODIFIED OLIGONUCLEOTIDES FOR USE IN RNA INTERFERENCE**

### **Field of the Invention**

          The present invention relates to the use of modified oligonucleotides that inhibit gene expression. In preferred embodiments the modified oligonucleotides  
10   modulate gene expression using the RNA interference pathway. More specifically, selected positions of the oligonucleotides are modified to give modified nucleosides that mimic RNA's 3'-endo sugar conformation. Preferred modifications include 2'-substituent groups and heterocyclic base modifications. The use of these modified oligonucleotides having 5'-phosphate groups is also  
15   disclosed.

### **Background of the Invention**

          In many species, introduction of double-stranded RNA (dsRNA) induces potent and specific gene silencing. This phenomenon occurs in both plants and  
20   animals and has roles in viral defense and transposon silencing mechanisms. This phenomenon was originally described more than a decade ago by researchers working with the petunia flower. While trying to deepen the purple color of these flowers, Jorgensen et al. introduced a pigment-producing gene under the control of a powerful promoter. Instead of the expected deep purple color, many of the  
25   flowers appeared variegated or even white. Jorgensen named the observed phenomenon "cosuppression", since the expression of both the introduced gene and the homologous endogenous gene was suppressed (Napoli et al., *Plant Cell*, **1990**, 2, 279-289; Jorgensen et al., *Plant Mol. Biol.*, **1996**, 31, 957-973).

          Cosuppression has since been found to occur in many species of plants,  
30   fungi, and has been particularly well characterized in *Neurospora crassa*, where it is known as "quelling" (Cogoni and Macino, *Genes Dev.* **2000**, 10, 638-643;

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Guru, *Nature*, **2000**, 404, 804-808).

The first evidence that dsRNA could lead to gene silencing in animals came from work in the nematode, *Caenorhabditis elegans*. In 1995, researchers Guo and Kemphues were attempting to use antisense RNA to shut down  
5 expression of the par-1 gene in order to assess its function. As expected, injection of the antisense RNA disrupted expression of par-1, but quizzically, injection of the sense-strand control also disrupted expression (Guo and Kempheus, *Cell*, **1995**, 81, 611-620). This result was a puzzle until Fire et al. injected dsRNA (a mixture of both sense and antisense strands) into *C. elegans*. This injection  
10 resulted in much more efficient silencing than injection of either the sense or the antisense strands alone. Injection of just a few molecules of dsRNA per cell was sufficient to completely silence the homologous gene's expression. Furthermore, injection of dsRNA into the gut of the worm caused gene silencing not only throughout the worm, but also in first generation offspring (Fire et al., *Nature*,  
15 **1998**, 391, 806-811).

The potency of this phenomenon led Timmons and Fire to explore the limits of the dsRNA effects by feeding nematodes bacteria that had been engineered to express dsRNA homologous to the *C. elegans* unc-22 gene. Surprisingly, these worms developed an unc-22 null-like phenotype (Timmons  
20 and Fire, *Nature* **1998**, 395, 854; Timmons et al., *Gene*, **2001**, 263, 103-112). Further work showed that soaking worms in dsRNA was also able to induce silencing (Tabara et al., *Science*, **1998**, 282, 430-431). PCT publication WO 01/48183 discloses methods of inhibiting expression of a target gene in a nematode worm involving feeding to the worm a food organism which is capable  
25 of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a portion of the target gene following ingestion of the food organism by the nematode, or by introducing a DNA capable of producing the double-stranded RNA structure (Bogaert et al., 2001).

The posttranscriptional gene silencing defined in *Caenorhabditis elegans*  
30 resulting from exposure to double-stranded RNA (dsRNA) has since been designated as RNA interference (RNAi). This term has come to generalize all

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forms of gene silencing involving dsRNA leading to the sequence-specific reduction of endogenous targeted mRNA levels; unlike co-suppression, in which transgenic DNA leads to silencing of both the transgene and the endogenous gene.

Introduction of exogenous double-stranded RNA (dsRNA) into

5 *Caenorhabditis elegans* has been shown to specifically and potently disrupt the activity of genes containing homologous sequences. Montgomery et al. suggests that the primary interference effects of dsRNA are post-transcriptional; this conclusion being derived from examination of the primary DNA sequence after dsRNA-mediated interference a finding of no evidence of alterations followed by  
10 studies involving alteration of an upstream operon having no effect on the activity of its downstream gene. These results argue against an effect on initiation or elongation of transcription. Finally they observed by in situ hybridization, that dsRNA-mediated interference produced a substantial, although not complete, reduction in accumulation of nascent transcripts in the nucleus, while cytoplasmic  
15 accumulation of transcripts was virtually eliminated. These results indicate that the endogenous mRNA is the primary target for interference and suggest a mechanism that degrades the targeted mRNA before translation can occur. It was also found that this mechanism is not dependent on the SMG system, an mRNA surveillance system in *C. elegans* responsible for targeting and destroying aberrant  
20 messages. The authors further suggest a model of how dsRNA might function as a catalytic mechanism to target homologous mRNAs for degradation. (Montgomery et al., *Proc. Natl. Acad. Sci. U S A*, 1998, 95, 15502-15507).

Recently, the development of a cell-free system from syncytial blastoderm *Drosophila* embryos that recapitulates many of the features of RNAi has been  
25 reported. The interference observed in this reaction is sequence specific, is promoted by dsRNA but not single-stranded RNA, functions by specific mRNA degradation, and requires a minimum length of dsRNA. Furthermore, preincubation of dsRNA potentiates its activity demonstrating that RNAi can be mediated by sequence-specific processes in soluble reactions (Tuschl et al., *Genes*  
30 *Dev.*, 1999, 13, 3191-3197).

In subsequent experiments, Tuschl et al, using the *Drosophila* in vitro



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system, demonstrated that 21- and 22-nt RNA fragments are the sequence-specific mediators of RNAi. These fragments, which they termed short interfering RNAs (siRNAs) were shown to be generated by an RNase III-like processing reaction from long dsRNA. They also showed that chemically synthesized siRNA duplexes with overhanging 3' ends mediate efficient target RNA cleavage in the *Drosophila* lysate, and that the cleavage site is located near the center of the region spanned by the guiding siRNA. In addition, they suggest that the direction of dsRNA processing determines whether sense or antisense target RNA can be cleaved by the siRNA-protein complex (Elbashir et al., *Genes Dev.*, **2001**, *15*, 188-200).

Further characterization of the suppression of expression of endogenous and heterologous genes caused by the 21-23 nucleotide siRNAs have been investigated in several mammalian cell lines, including human embryonic kidney (293) and HeLa cells (Elbashir et al., *Nature*, **2001**, *411*, 494-498).

The *Drosophila* embryo extract system has been exploited, using green fluorescent protein and luciferase tagged siRNAs, to demonstrate that siRNAs can serve as primers to transform the target mRNA into dsRNA. The nascent dsRNA is degraded to eliminate the incorporated target mRNA while generating new siRNAs in a cycle of dsRNA synthesis and degradation. Evidence is also presented that mRNA-dependent siRNA incorporation to form dsRNA is carried out by an RNA-dependent RNA polymerase activity (RdRP) (Lipardi et al., *Cell*, **2001**, *107*, 297-307).

The involvement of an RNA-directed RNA polymerase and siRNA primers as reported by Lipardi et al. (Lipardi et al., *Cell*, **2001**, *107*, 297-307) is one of the many intriguing features of gene silencing by RNA interference; suggesting an apparent catalytic nature to the phenomenon. New biochemical and genetic evidence reported by Nishikura et al. also shows that an RNA-directed RNA polymerase chain reaction, primed by siRNA, amplifies the interference caused by a small amount of "trigger" dsRNA (Nishikura, *Cell*, **2001**, *107*, 415-418).

Investigating the role of "trigger" RNA amplification during RNA interference (RNAi) in *Caenorhabditis elegans*, Sijen et al revealed a substantial

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fraction of siRNAs that cannot derive directly from input dsRNA. Instead, a population of siRNAs (termed secondary siRNAs) appeared to derive from the action of the previously reported cellular RNA-directed RNA polymerase (RdRP) on mRNAs that are being targeted by the RNAi mechanism. The distribution of secondary siRNAs exhibited a distinct polarity (5'-3'; on the antisense strand), suggesting a cyclic amplification process in which RdRP is primed by existing siRNAs. This amplification mechanism substantially augmented the potency of RNAi-based surveillance, while ensuring that the RNAi machinery will focus on expressed mRNAs (Sijen et al., *Cell*, **2001**, *107*, 465-476).

Most recently, Tijsterman et al. have shown that, in fact, single-stranded RNA oligomers of antisense polarity can be potent inducers of gene silencing. As is the case for co-suppression, they showed that antisense RNAs act independently of the RNAi genes *rde-1* and *rde-4* but require the mutator/RNAi gene *mut-7* and a putative DEAD box RNA helicase, *mut-14*. According to the authors, their data favor the hypothesis that gene silencing is accomplished by RNA primer extension using the mRNA as template, leading to dsRNA that is subsequently degraded suggesting that single-stranded RNA oligomers are ultimately responsible for the RNAi phenomenon (Tijsterman et al., *Science*, **2002**, *295*, 694-697).

Several recent publications have described the structural requirements for the dsRNA trigger required for RNAi activity. Recent reports have indicated that ideal dsRNA sequences are 21nt in length containing 2 nt 3'-end overhangs (Elbashir et al, EMBO (2001), *20*, 6877-6887, Sabine Brantl, *Biochimica et Biophysica Acta*, **2002**, *1575*, 15-25.) In this system, substitution of the 4 nucleosides from the 3'-end with 2'-deoxynucleosides has been demonstrated to not affect activity. On the other hand, substitution with 2'-deoxynucleosides or 2'-OMe-nucleosides throughout the sequence (sense or antisense) was shown to be deleterious to RNAi activity. Investigation of the structural requirements for RNA silencing in *C. elegans* has demonstrated modification of the internucleotide linkage (phosphorothioate) to not interfere with activity (Parrish et al, *Molecular Cell* (2000), *6*, 1077-1087.) It was also shown by Parrish *et al.*, that chemical modification like

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2'-amino or 5'-iodouridine are well tolerated in the sense strand but not the antisense strand of the dsRNA suggesting differing roles for the 2 strands in RNAi. Base modification such as guanine to inosine (where one hydrogen bond is lost) has been demonstrated to decrease RNAi activity independently of the position of the modification (sense or antisense). Same "position independent" loss of activity has been observed following the introduction of mismatches in the dsRNA trigger. Some types of modifications, for example introduction of sterically demanding bases such as 5-iodoU, have been shown to be deleterious to RNAi activity when positioned in the antisense strand, whereas modifications positioned in the sense strand were shown to be less detrimental to RNAi activity. As was the case for the 21 nt dsRNA sequences, RNA-DNA heteroduplexes did not serve as triggers for RNAi. However, dsRNA containing 2'-F-2'-deoxynucleosides appeared to be efficient in triggering RNAi response independent of the position (sense or antisense) of the 2'-F-2'-deoxynucleosides.

In one study the reduction of gene expression was studied using electroporated dsRNA and a 25mer morpholino in post implantation mouse embryos (Mellitzer *et al.*, *Mechanisms of Development*, **2002**, 118, 57-63). The morpholino oligomer did show activity but was not as effective as the dsRNA.

A number of PCT applications have recently been published that relate to the RNAi phenomenon. These include: PCT publication WO 00/44895; PCT publication WO 00/49035; PCT publication WO 00/63364; PCT publication WO 01/36641; PCT publication WO 01/36646; PCT publication WO 99/32619; PCT publication WO 00/44914; PCT publication WO 01/29058; and PCT publication WO 01/75164.

U.S. patents 5,898,031 and 6,107,094, each of which is commonly owned with this application and each of which is herein incorporated by reference, describe certain oligonucleotide having RNA like properties. When hybridized with RNA, these oligonucleotides serve as substrates for a dsRNase enzyme with resultant cleavage of the RNA by the enzyme.

In another recently published paper (Martinez *et al.*, *Cell*, **2002**, 110, 563-574) it was shown that double stranded as well as single stranded siRNA resides in the RNA-

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induced silencing complex (RISC) together with eIF2C1 and eIF2C2 (human GERP950 Argonaute proteins. The activity of 5'-phosphorylated single stranded siRNA was comparable to the double stranded siRNA in the system studied.

In yet another recently published paper (Chiu *et al.*, *Molecular Cell*, **2002**, *10*, 549-561) it was shown that the 5'-hydroxyl group of the siRNA is essential as it is phosphorylated for activity while the 3'-hydroxyl group is not essential and tolerates substitute groups such as biotin. It was further shown that bulge structures in one or both of the sense or antisense strands either abolished or severely lowered the activity relative to the unmodified siRNA duplex. Also shown was severe lowering of activity when psoralen was used to cross link an siRNA duplex.

Like antisense siRNA technology is an effective means for modulating the levels of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications involving gene silencing. The present invention therefore further provides oligonucleotides useful for modulating gene silencing pathways, including those involving antisense, RNA interference, dsRNA enzymes and non-antisense mechanisms. One having skill in the art, once armed with this disclosure will be able, without undue experimentation, to identify preferred oligonucleotide compounds for these uses.

20

### Summary of the Invention

In one embodiment, the invention relates to a method of inhibiting the expression of a gene in a biological system expressing said gene, comprising the steps of:

25 providing a composition comprising a modified siRNA; and  
contacting said biological system with said modified siRNA under conditions effective to inhibit the expression of said gene;

said modified siRNA comprising at least one RNA strand having at least one modified nucleoside wherein said modified nucleoside has enhanced binding affinity relative to the unmodified RNA nucleoside in the same position.

30

In certain preferred embodiments, the sugar moiety of said modified nucleoside

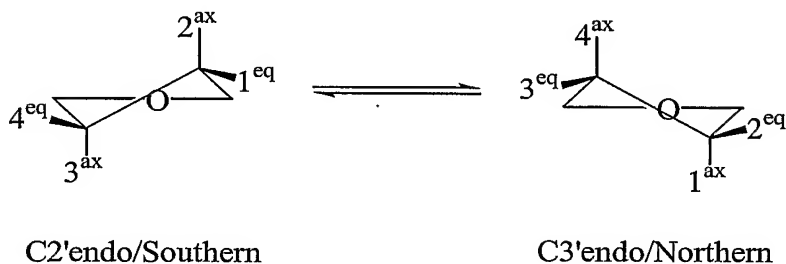
- 8 -

has 3'-endo conformational geometry.

### Detailed Description of the Invention

The present invention provides modified oligonucleotides useful in the RNAi pathway. The oligonucleotides of the invention are modified by having at least one structurally modified nucleoside. The structurally modified nucleosides mimic RNA by having 3'-endo conformational geometry. The use of modified oligonucleotides enables a wider variety of chemistries that have advantages over native RNA such as but not limited to modulation of pharmacokinetic properties through modification of protein binding, protein off-rate, absorption and clearance; modulation of nuclease stability as well as chemical stability; modulation of the binding affinity and specificity of the oligomer (affinity and specificity for enzymes as well as for complementary sequences); and increasing efficacy of RNA cleavage.

The apparent preference for an RNA type duplex (A form helix, predominantly 3'-endo) as a trigger of the RNAi response is further supported by the fact that duplexes composed of 2'-deoxy-2'-F-nucleosides appears efficient in triggering RNAi response in the *C. elegans* system. Based on these observations, this invention provides oligomeric triggers of RNAi having one or more nucleosides modified in such a way as to favor a C3'-endo type conformation (see Scheme 1 below.)



**Scheme 1**

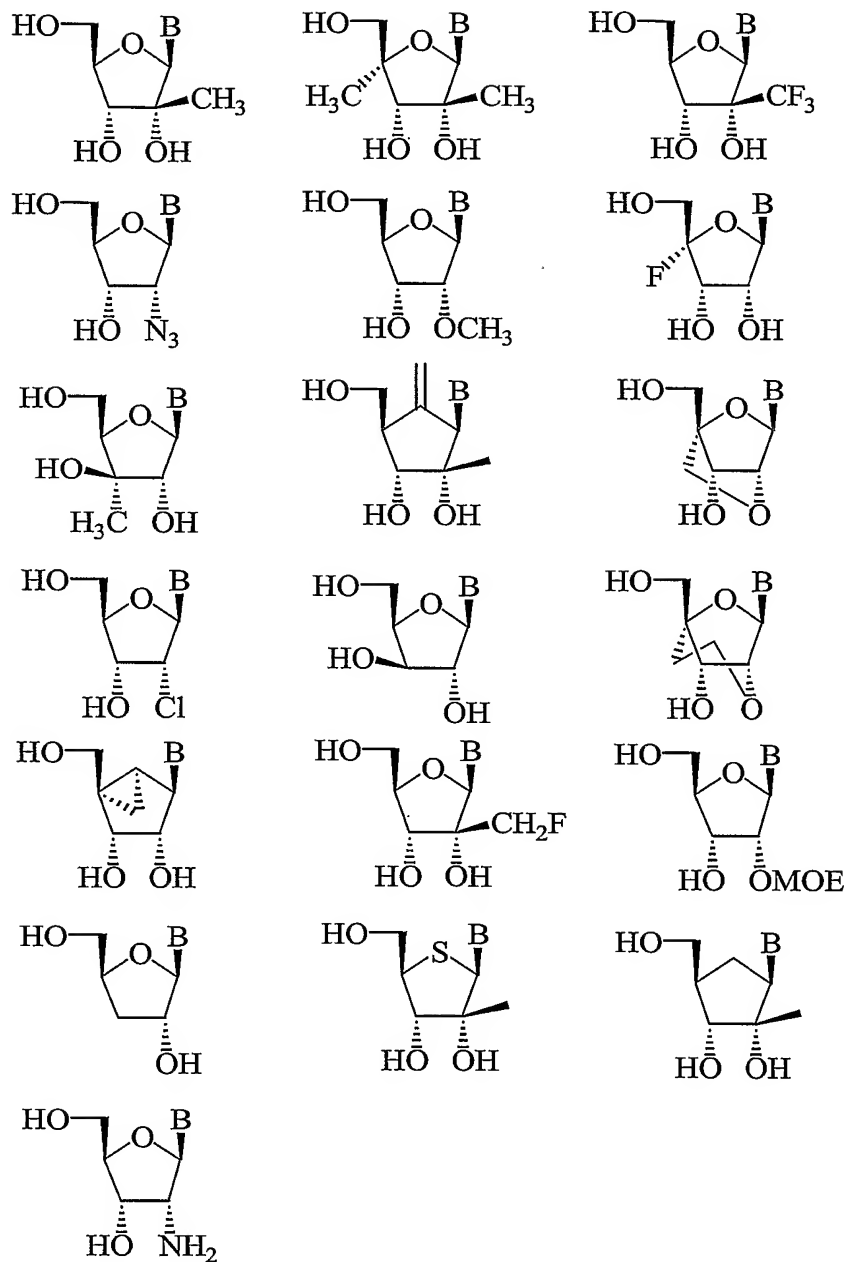
Nucleoside conformation is influenced by various factors including substitution at the 2' or 3' positions. Electronegative substituents generally prefer the axial positions, while sterically demanding substituents generally prefer the equatorial positions (Principles of Nucleic Acid Structure, Wolfgang Sanger, 1984,

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Springer-Verlag.) Modification of the 2' position to favor the 3'-endo conformation can be achieved while maintaining the 2'-OH as a recognition element, as illustrated in Figure 2, below (Gallo et al., Tetrahedron (2001), 57, 5707-5713. Harry-O'kuru et al., J. Org. Chem., (1997), 62(6), 1754-1759 and Tang et al., J. Org. Chem. (1999), 64, 747-754.) Alternatively, preference for the 3'-endo conformation can be achieved by deletion of the 2'-OH as exemplified by 2'deoxy-2'F-nucleosides (Kawasaki et al., J. Med. Chem. (1993), 36, 831-841), which adopts the 3'-endo conformation positioning the electronegative fluorine atom in the axial position. Other modifications of the ribose ring, for example substitution at the 4'-position to give 4'-F modified nucleosides (Guillerm et al., Bioorganic and Medicinal Chemistry Letters (1995), 5, 1455-1460 and Owen et al., J. Org. Chem. (1976), 41, 3010-3017), or for example modification to yield methanocarpa nucleoside analogs (Jacobson et al., J. Med. Chem. Lett. (2000), 43, 2196-2203 and Lee et al., Bioorganic and Medicinal Chemistry Letters (2001), 11, 1333-1337) also induce preference for the 3'-endo conformation. Along similar lines, oligomeric triggers of RNAi response might be composed of one or more nucleosides modified in such a way that conformation is locked into a C3'-endo type conformation, i.e. Locked Nucleic Acid (LNA, Singh et al, Chem. Commun. (1998), 4, 455-456), and ethylene bridged Nucleic Acids (ENA, Morita et al, Bioorganic & Medicinal Chemistry Letters (2002), 12, 73-76.) Examples of modified nucleosides amenable to the present invention are shown below in Table 2. These examples are meant to be representative and not exhaustive.

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Table I



5           The preferred conformation of modified nucleosides and their oligomers can be estimated by various methods such as molecular dynamics calculations, nuclear magnetic resonance spectroscopy and CD measurements. Hence, modifications predicted to induce RNA like conformations, A-form duplex

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geometry in an oligomeric context, are selected for use in the modified oligonucleotides of the present invention. The synthesis of numerous of the modified nucleosides amenable to the present invention are known to the art skilled (see for example, Chemistry of Nucleosides and Nucleotides Vol 1-3, ed. Leroy B. Townsend, 1988, Plenum press.) Nucleosides known to be inhibitors/substrates for RNA dependent RNA polymerases (for example HCV NS5B) might be of particular interest in this context, and reference is made to the synthesis of such nucleosides (see PCT publications WO 02/57425 and WO 02/57287.) Oligomerization of modified and unmodified nucleosides will be performed according to literature procedures for DNA (Protocols for Oligonucleotides and Analogs, Ed. Agrawal (1993), Humana Press) and/or RNA (Scaringe, Methods (2001), 23, 206-217. Gait et al., Applications of Chemically synthesized RNA in RNA:Protein Interactions, Ed. Smith (1998), 1-36. Gallo et al., Tetrahedron (2001), 57, 5707-5713) synthesis as appropriate. Effect of nucleoside modifications on RNAi activity will be evaluated according to existing literature (Elbashir et al., Nature (2001), 411, 494-498; Nishikura et al., Cell (2001), 107, 415-416; and Bass et al., Cell (2000), 101, 235-238.)

In one aspect, the present invention is directed to oligonucleotides that are prepared having enhanced properties compared to native RNA against nucleic acid targets. A target is identified and an oligonucleotide is selected having an effective length and sequence that is preferably complementary to a portion of the target sequence. Each nucleoside of the selected sequence is scrutinized for possible enhancing modifications. A preferred modification would be the replacement of one or more RNA nucleosides with nucleosides that have the same 3'-endo conformational geometry. Such modifications can enhance chemical and nuclease stability relative to native RNA while at the same time being much cheaper and easier to synthesize and/or incorporate into an oligonucleotide. The selected sequence can be further divided into regions and the nucleosides of each region evaluated for enhancing modifications that can be the result of a chimeric configuration. Consideration is also given to the 5' and 3'-termini as there are often advantageous modifications that can be made to one or more of the terminal



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nucleosides. A preferred modification is a 5'-phosphate group as it can enhance the activity of the oligonucleotides of the invention. Further modifications are also considered such as internucleoside linkages, conjugate groups, substitute sugars or bases, substitution of one or more nucleosides with nucleoside mimetics  
5 and any other modification that can enhance the selected sequence for its intended target.

Nucleosides, a preferred monomeric subunit, can be modified in a variety of ways such as by attachment of a substituent group or a conjugate group or by modifying the base or the sugar. Modification of the sugar the base or both  
10 simultaneously can have an effect on the sugar puckering. The sugar puckering plays a central role in determining the duplex conformational geometry between an oligonucleotide and its nucleic acid target. By controlling the sugar puckering independently at each position of an oligonucleotide the duplex geometry can be modulated to help maximize the resulting oligonucleotide's efficacy. Modulation  
15 of sugar geometry has been shown to enhance properties such as for example increased lipophilicity, binding affinity to target nucleic acid (e.g. mRNA), chemical stability and nuclease resistance.

The terms used to describe the conformational geometry of homoduplex nucleic acids are "A Form" for RNA and "B Form" for DNA. The respective  
20 conformational geometry for RNA and DNA duplexes was determined from X-ray diffraction analysis of nucleic acid fibers (Arnott and Hukins, *Biochem. Biophys. Res. Comm.*, **1970**, *47*, 1504.) In general, RNA:RNA duplexes are more stable and have higher melting temperatures (T<sub>m</sub>) than DNA:DNA duplexes (Sanger et al., *Principles of Nucleic Acid Structure*, 1984, Springer-Verlag; New  
25 York, NY.; Lesnik et al., *Biochemistry*, 1995, *34*, 10807-10815; Conte et al., *Nucleic Acids Res.*, 1997, *25*, 2627-2634). The increased stability of RNA has been attributed to several structural features, most notably the improved base stacking interactions that result from an A-form geometry (Searle et al., *Nucleic Acids Res.*, 1993, *21*, 2051-2056). The presence of the 2' hydroxyl in RNA biases  
30 the sugar toward a C3' endo pucker, i.e., also designated as Northern pucker, which causes the duplex to favor the A-form geometry. In addition, the 2'

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hydroxyl groups of RNA can form a network of water mediated hydrogen bonds that help stabilize the RNA duplex (Egli et al., *Biochemistry*, 1996, 35, 8489-8494). On the other hand, deoxy nucleic acids prefer a C2' endo sugar pucker, i.e., also known as Southern pucker, which is thought to impart a less stable B-form geometry (Sanger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York, NY). As used herein, B-form geometry is inclusive of both C2'-endo pucker and O4'-endo pucker. This is consistent with Berger, *et. al.*, *Nucleic Acids Research*, 1998, 26, 2473-2480, who pointed out that in considering the furanose conformations which give rise to B-form duplexes consideration should also be given to a O4'-endo pucker contribution.

DNA:RNA hybrid duplexes, however, are usually less stable than pure RNA:RNA duplexes, and depending on their sequence may be either more or less stable than DNA:DNA duplexes (Searle *et al.*, *Nucleic Acids Res.*, 1993, 21, 2051-2056). The structure of a hybrid duplex is intermediate between A- and B-form geometries, which may result in poor stacking interactions (Lane *et al.*, *Eur. J. Biochem.*, 1993, 215, 297-306; Fedoroff *et al.*, *J. Mol. Biol.*, 1993, 233, 509-523; Gonzalez *et al.*, *Biochemistry*, 1995, 34, 4969-4982; Horton *et al.*, *J. Mol. Biol.*, 1996, 264, 521-533). The stability of the duplex formed between a target RNA and a synthetic sequence is central to therapies such as but not limited to antisense and RNA interference as these mechanisms require the binding of a synthetic oligonucleotide strand to an RNA target strand. In the case of antisense, effective inhibition of the mRNA requires that the antisense DNA have a very high binding affinity with the mRNA. Otherwise the desired interaction between the synthetic oligonucleotide strand and target mRNA strand will occur infrequently, resulting in decreased efficacy.

One routinely used method of modifying the sugar puckering is the substitution of the sugar at the 2'-position with a substituent group that influences the sugar geometry. The influence on ring conformation is dependant on the nature of the substituent at the 2'-position. A number of different substituents have been studied to determine their sugar puckering effect. For example, 2'-halogens have been studied showing that the 2'-fluoro derivative exhibits the

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largest population (65%) of the C3'-endo form, and the 2'-iodo exhibits the lowest population (7%). The populations of adenosine (2'-OH) versus deoxyadenosine (2'-H) are 36% and 19%, respectively. Furthermore, the effect of the 2'-fluoro group of adenosine dimers (2'-deoxy-2'-fluoroadenosine - 2'-deoxy-2'-fluoro-  
5 adenosine) is further correlated to the stabilization of the stacked conformation.

As expected, the relative duplex stability can be enhanced by replacement of 2'-OH groups with 2'-F groups thereby increasing the C3'-endo population. It is assumed that the highly polar nature of the 2'-F bond and the extreme preference for C3'-endo puckering may stabilize the stacked conformation in an A-form  
10 duplex. Data from UV hypochromicity, circular dichroism, and <sup>1</sup>H NMR also indicate that the degree of stacking decreases as the electronegativity of the halo substituent decreases. Furthermore, steric bulk at the 2'-position of the sugar moiety is better accommodated in an A-form duplex than a B-form duplex. Thus, a 2'-substituent on the 3'-terminus of a dinucleoside monophosphate is thought to  
15 exert a number of effects on the stacking conformation: steric repulsion, furanose puckering preference, electrostatic repulsion, hydrophobic attraction, and hydrogen bonding capabilities. These substituent effects are thought to be determined by the molecular size, electronegativity, and hydrophobicity of the substituent. Melting temperatures of complementary strands is also increased  
20 with the 2'-substituted adenosine diphosphates. It is not clear whether the 3'-endo preference of the conformation or the presence of the substituent is responsible for the increased binding. However, greater overlap of adjacent bases (stacking) can be achieved with the 3'-endo conformation.

One synthetic 2'-modification that imparts increased nuclease resistance  
25 and a very high binding affinity to nucleotides is the 2-methoxyethoxy (2'-MOE, 2'-OCH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>) side chain (Baker *et al.*, *J. Biol. Chem.*, **1997**, 272, 11944-12000). One of the immediate advantages of the 2'-MOE substitution is the improvement in binding affinity, which is greater than many similar 2' modifications such as *O*-methyl, *O*-propyl, and *O*-aminopropyl. Oligonucleotides  
30 having the 2'-*O*-methoxyethyl substituent also have been shown to be antisense inhibitors of gene expression with promising features for *in vivo* use (Martin, P.,

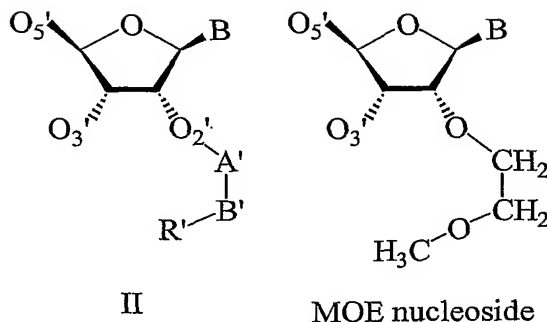
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*Helv. Chim. Acta*, **1995**, 78, 486-504; Altmann *et al.*, *Chimia*, **1996**, 50, 168-176; Altmann *et al.*, *Biochem. Soc. Trans.*, **1996**, 24, 630-637; and Altmann *et al.*, *Nucleosides Nucleotides*, 1997, **16**, 917-926). Relative to DNA, the oligonucleotides having the 2'-MOE modification displayed improved RNA  
5 affinity and higher nuclease resistance. Chimeric oligonucleotides having 2'-MOE substituents in the wing nucleosides and an internal region of deoxy-phosphorothioate nucleotides (also termed a gapped oligonucleotide or gapmer) have shown effective reduction in the growth of tumors in animal models at low doses. 2'-MOE substituted oligonucleotides have also shown outstanding promise  
10 as antisense agents in several disease states. One such MOE substituted oligonucleotide is presently being investigated in clinical trials for the treatment of CMV retinitis.

To better understand the higher RNA affinity of 2'-*O*-methoxyethyl substituted RNA and to examine the conformational properties of the 2'-*O*-  
15 methoxyethyl substituent, two dodecamer oligonucleotides were synthesized having SEQ ID NO: 1 (CGC GAA UUC GCG) and SEQ ID NO: 2 (GCG CUU AAG CGC). These self-complementary strands have every 2'-position modified with a 2'-*O*-methoxyethyl. The duplex was crystallized at a resolution of 1.7 Ångstrom and the crystal structure was determined. The conditions used for the  
20 crystallization were 2 mM oligonucleotide, 50 mM Na Hepes pH 6.2-7.5, 10.50 mM MgCl<sub>2</sub>, 15% PEG 400. The crystal data showed: space group C2, cell constants  $a=41.2$  Å,  $b=34.4$  Å,  $c=46.6$  Å,  $\beta=92.4^\circ$ . The resolution was 1.7 Å at -170°C. The current  $R$ -factor was 20% ( $R_{\text{free}}$  26%).

This crystal structure is believed to be the first crystal structure of a fully  
25 modified RNA oligonucleotide analogue. The duplex adopts an overall A-form conformation and all modified sugars display C3'-*endo* pucker. In most of the 2'-*O*-substituents, the torsion angle around the A'-B' bond, as depicted in Structure II below, of the ethylene glycol linker has a *gauche* conformation. For 2'-*O*-MOE, A' and B' of Structure II below are methylene moieties of the ethyl portion of the  
30 MOE and R' is the methoxy portion.

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In the crystal, the 2'-O-MOE RNA duplex adopts a general orientation such that the crystallographic 2-fold rotation axis does not coincide with the molecular 2-fold rotation axis. The duplex adopts the expected A-type geometry and all of the 24 2'-O-MOE substituents were visible in the electron density maps at full resolution. The electron density maps as well as the temperature factors of substituent atoms indicate flexibility of the 2'-O-MOE substituent in some cases.

Most of the 2'-O-MOE substituents display a *gauche* conformation around the C-C bond of the ethyl linker. However, in two cases, a *trans* conformation around the C-C bond is observed. The lattice interactions in the crystal include packing of duplexes against each other via their minor grooves. Therefore, for some residues, the conformation of the 2'-O-substituent is affected by contacts to an adjacent duplex. In general, variations in the conformation of the substituents (*e.g.*  $g^+$  or  $g^-$  around the C-C bonds) create a range of interactions between substituents, both inter-strand, across the minor groove, and intra-strand. At one location, atoms of substituents from two residues are in van der Waals contact across the minor groove. Similarly, a close contact occurs between atoms of substituents from two adjacent intra-strand residues.

Previously determined crystal structures of A-DNA duplexes were for those that incorporated isolated 2'-O-methyl T residues. In the crystal structure noted above for the 2'-O-MOE substituents, a conserved hydration pattern has been observed for the 2'-O-MOE residues. A single water molecule is seen located between O2', O3' and the methoxy oxygen atom of the substituent, forming contacts to all three of between 2.9 and 3.4 Å. In addition, oxygen atoms of substituents are involved in several other hydrogen bonding contacts. For

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example, the methoxy oxygen atom of a particular 2'-O-substituent forms a hydrogen bond to N3 of an adenosine from the opposite strand via a bridging water molecule.

In several cases a water molecule is trapped between the oxygen atoms  
5 O2', O3' and OC' of modified nucleosides. 2'-O-MOE substituents with *trans* conformation around the C-C bond of the ethylene glycol linker are associated with close contacts between OC' and N2 of a guanosine from the opposite strand, and, water-mediated, between OC' and N3(G). When combined with the available thermodynamic data for duplexes containing 2'-O-MOE modified strands, this  
10 crystal structure allows for further detailed structure-stability analysis of other antisense modifications.

In extending the crystallographic structure studies, molecular modeling experiments were performed to study further enhanced binding affinity of oligonucleotides having 2'-O-modifications of the invention. The computer  
15 simulations were conducted on compounds of SEQ ID NO: 1, above, having 2'-O-modifications of the invention located at each of the nucleoside of the oligonucleotide. The simulations were performed with the oligonucleotide in aqueous solution using the AMBER force field method (Cornell *et al.*, *J. Am. Chem. Soc.*, **1995**, *117*, 5179-5197)(modeling software package from UCSF, San  
20 Francisco, CA). The calculations were performed on an Indigo2 SGI machine (Silicon Graphics, Mountain View, CA).

Further 2'-O-modifications of the inventions include those having a ring structure that incorporates a two atom portion corresponding to the A' and B' atoms of Structure II. The ring structure is attached at the 2' position of a sugar  
25 moiety of one or more nucleosides that are incorporated into an oligonucleotide. The 2'-oxygen of the nucleoside links to a carbon atom corresponding to the A' atom of Structure II. These ring structures can be aliphatic, unsaturated aliphatic, aromatic or heterocyclic. A further atom of the ring (corresponding to the B' atom of Structure II), bears a further oxygen atom, or a sulfur or nitrogen atom. This  
30 oxygen, sulfur or nitrogen atom is bonded to one or more hydrogen atoms, alkyl moieties, or haloalkyl moieties, or is part of a further chemical moiety such as a

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ureido, carbamate, amide or amidine moiety. The remainder of the ring structure restricts rotation about the bond joining these two ring atoms. This assists in positioning the "further oxygen, sulfur or nitrogen atom" (part of the R position as described above) such that the further atom can be located in close proximity to the 3'-oxygen atom (O3') of the nucleoside.

Another 2'-substituent that has been studied is the 2'-OMe group. 2'-Substitution of guanosine, cytidine, and uridine dinucleoside phosphates with the 2'-OMe group showed enhanced stacking effects with respect to the corresponding native (2'-OH) species leading to the conclusion that the sugar is adopting a C3'-endo conformation. In this case, it is believed that the hydrophobic attractive forces of the methyl group tend to overcome the destabilizing effects of its steric bulk.

The ability of oligonucleotides to bind to their complementary target strands is compared by determining the melting temperature ( $T_m$ ) of the hybridization complex of the oligonucleotide and its complementary strand. The melting temperature ( $T_m$ ), a characteristic physical property of double helices, denotes the temperature (in degrees centigrade) at which 50% helical (hybridized) versus coil (unhybridized) forms are present.  $T_m$  is measured by using the UV spectrum to determine the formation and breakdown (melting) of the hybridization complex. Base stacking, which occurs during hybridization, is accompanied by a reduction in UV absorption (hypochromicity). Consequently, a reduction in UV absorption indicates a higher  $T_m$ . The higher the  $T_m$ , the greater the strength of the bonds between the strands.

Freier and Altmann, *Nucleic Acids Research*, (1997) 25:4429-4443, have previously published a study on the influence of structural modifications of oligonucleotides on the stability of their duplexes with target RNA. In this study, the authors reviewed a series of oligonucleotides containing more than 200 different modifications that had been synthesized and assessed for their hybridization affinity and  $T_m$ . Sugar modifications studied included substitutions on the 2'-position of the sugar, 3'-substitution, replacement of the 4'-oxygen, the use of bicyclic sugars, and four member ring replacements. Several nucleobase

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modifications were also studied including substitutions at the 5, or 6 position of thymine, modifications of pyrimidine heterocycle and modifications of the purine heterocycle. Modified internucleoside linkages were also studied including neutral, phosphorus and non-phosphorus containing internucleoside linkages.

5           Four general approaches might be used to improve hybridization of oligonucleotides to RNA targets. These include: preorganization of the sugars and phosphates of the oligodeoxynucleotide strand into conformations favorable for hybrid formation, improving stacking of nucleobases by the addition of polarizable groups to the heterocycle bases of the nucleotides of the  
10           oligonucleotide, increasing the number of H-bonds available for A-U pairing, and neutralization of backbone charge to facilitate removing undesirable repulsive interactions. It was found that utilizing the first of these, preorganization of the sugars and phosphates of the oligonucleotide strand into conformations favorable for hybrid formation, is a preferred method to achieve improved binding affinity.  
15           It can further be used in combination with one or more of the other three approaches.

          Increasing the percentage of C3'-endo sugars in a modified oligonucleotide targeted to an RNA target strand should preorganize this strand for binding to RNA. Of the several sugar modifications that have been reported and studied in  
20           the literature, the incorporation of electronegative substituents such as 2'-fluoro or 2'-alkoxy shift the sugar conformation towards the 3' endo (northern) pucker conformation. This preorganizes an oligonucleotide that incorporates such modifications to have an A-form conformational geometry. This A-form conformation results in increased binding affinity of the oligonucleotide to a target  
25           RNA strand.

          Molecular modeling experiments were performed to study further enhanced binding affinity of oligonucleotides having 2'-O-modifications. Computer simulations were conducted on compounds having SEQ ID NO: 1 with various 2'-O-modifications located at each of the nucleosides of the  
30           oligonucleotide. The simulations were performed with the oligonucleotide in aqueous solution using the AMBER force field method (Cornell *et al.*, *J. Am.*



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*Chem. Soc.*, 1995, 117, 5179-5197)(modeling software package from UCSF, San Francisco, CA). The calculations were performed on an Indigo2 SGI machine (Silicon Graphics, Mountain View, CA).

In addition, for 2'-substituents containing an ethylene glycol motif, a  
5 *gauche* interaction between the oxygen atoms around the O-C-C-O torsion of the side chain may have a stabilizing effect on the duplex (Freier *ibid.*). Such *gauche* interactions have been observed experimentally for a number of years (Wolfe *et al.*, *Acc. Chem. Res.*, 1972, 5, 102; Abe *et al.*, *J. Am. Chem. Soc.*, 1976, 98, 468). This *gauche* effect may result in a configuration of the side chain that is favorable  
10 for duplex formation. The exact nature of this stabilizing configuration has not yet been explained. While we do not want to be bound by theory, it may be that holding the O-C-C-O torsion in a single *gauche* configuration, rather than a more random distribution seen in an alkyl side chain, provides an entropic advantage for duplex formation.

15 Representative 2'-substituent groups amenable to the present invention that improve binding affinity and are thought to configure the sugar group to which they are attached into a 3'-endo conformational geometry include 2'-O-alkyl, 2'-O-substituted alkyl and 2'-fluoro substituent groups. Preferred for the substituent groups are various alkyl and aryl ethers and thioethers, amines and monoalkyl and  
20 dialkyl substituted amines. It is further intended that multiple modifications can be made to one or more nucleosides and or internucleoside linkages within an oligonucleotide of the invention to enhance the activity and or desired properties of the oligonucleotide. Tables I through VII list nucleoside and internucleoside linkage modifications/replacements that have been shown to give a positive  $\Delta T_m$   
25 per modification when the modification/replacement was made to a DNA strand that was hybridized to an RNA complement.

Table I

**Modified DNA strand having 2'-substituent groups that gave an overall increase in T<sub>m</sub> against an RNA complement:**

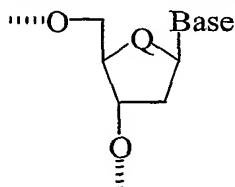
5	<b><u>Positive <math>\Delta T_m</math>/mod</u></b>
	<b>2'-substituents</b>
	2'-OH
	2'-O-C <sub>1</sub> -C <sub>4</sub> alkyl
	2'-O-(CH <sub>2</sub> ) <sub>2</sub> CH <sub>3</sub>
	2'-O-CH <sub>2</sub> CH=CH <sub>2</sub>
10	2'-F
	2'-O-(CH <sub>2</sub> ) <sub>2</sub> -O-CH <sub>3</sub>
	2'-[O-(CH <sub>2</sub> ) <sub>2</sub> ] <sub>2</sub> -O-CH <sub>3</sub>
	2'-[O-(CH <sub>2</sub> ) <sub>2</sub> ] <sub>3</sub> -O-CH <sub>3</sub>
	2'-[O-(CH <sub>2</sub> ) <sub>2</sub> ] <sub>4</sub> -O-CH <sub>3</sub>
15	2'-[O-(CH <sub>2</sub> ) <sub>2</sub> ] <sub>3</sub> -O-(CH <sub>2</sub> ) <sub>8</sub> CH <sub>3</sub>
	2'-O-(CH <sub>2</sub> ) <sub>2</sub> CF <sub>3</sub>
	2'-O-(CH <sub>2</sub> ) <sub>2</sub> OH
	2'-O-(CH <sub>2</sub> ) <sub>2</sub> F
	2'-O-CH <sub>2</sub> CH(CH <sub>3</sub> )F
20	2'-O-CH <sub>2</sub> CH(CH <sub>2</sub> OH)OH
	2'-O-CH <sub>2</sub> CH(CH <sub>2</sub> OCH <sub>3</sub> )OCH <sub>3</sub>
	2'-O-CH <sub>2</sub> CH(CH <sub>3</sub> )OCH <sub>3</sub>
	2'-O-CH <sub>2</sub> -C <sub>14</sub> H <sub>7</sub> O <sub>2</sub> (-C <sub>14</sub> H <sub>7</sub> O <sub>2</sub> = Anthraquinone)
	2'-O-(CH <sub>2</sub> ) <sub>3</sub> -NH <sub>2</sub> *
25	2'-O-(CH <sub>2</sub> ) <sub>4</sub> -NH <sub>2</sub> *

\* These modifications can increase or decrease the relative T<sub>m</sub> dependent on the number of modifications and their position (motif dependant).

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Table II

Modified DNA strand having modified sugar ring (see structure x) that give an overall increase in  $T_m$  against an RNA complement:



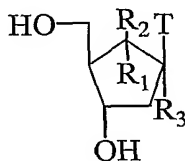
5 Positive  $\Delta T_m/\text{mod}$

Q -S-  
-CH<sub>2</sub>-

Note: In general ring oxygen substitution with sulfur or methylene had only a minor effect on  $T_m$  for the specific motifs studied. Substitution at the 2'-  
10 position with groups shown to stabilize the duplex were destabilizing when CH<sub>2</sub> replaced the ring O. This is thought to be due to the necessary gauche interaction between the ring O with particular 2'-substituents (for example -O-CH<sub>3</sub> and -(O-CH<sub>2</sub>CH<sub>2</sub>)<sub>3</sub>-O-CH<sub>3</sub>).

15 Table III

Modified DNA strand having modified sugar ring that give an overall increase in  $T_m$  against an RNA complement:



20

Positive  $\Delta T_m/\text{mod}$

-C(R<sub>2</sub>)R<sub>1</sub> effects OH  
(R<sub>2</sub>, R<sub>3</sub> both = H) CH<sub>3</sub>\*  
CH<sub>2</sub>OH\*  
25 OCH<sub>3</sub>\*

\* These modifications can increase or decrease the relative  $T_m$  dependent

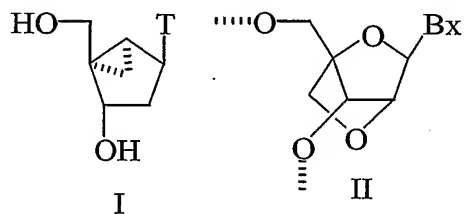
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on the number of modifications and their position (motiff dependant).

**Table IV**

**Modified DNA strand having bicyclic substitute sugar modifications**  
**that give an overall increase in Tm against an RNA complement:**

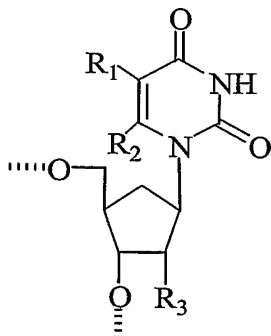
<b>Formula</b>	<b><u>Positive <math>\Delta T_m/\text{mod}</math></u></b>
I	+
II	+

**Table V**

**Modified DNA strand having modified heterocyclic base moieties that**  
**give an overall increase in Tm against an RNA complement:**

<b><u>Modification/Formula</u></b>	<b><u>Positive <math>\Delta T_m/\text{mod}</math></u></b>
Heterocyclic base	2-thioT
modifications	2'-O-methylpseudoU
	7-halo-7-deaza purines
	7-propyne-7-deaza purines
	2-aminoA(2,6-diaminopurine)

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Modification/FormulaPositive  $\Delta T_m/\text{mod}$ (R<sub>2</sub>, R<sub>3</sub>=H), R<sub>1</sub>=

Br

C≡C-CH<sub>3</sub>(CH<sub>2</sub>)<sub>3</sub>NH<sub>2</sub>CH<sub>3</sub>Motiffs-disubstitutionR<sub>1</sub>= C≡C-CH<sub>3</sub>, R<sub>2</sub>=H, R<sub>3</sub>=

F

R<sub>1</sub>= C≡C-CH<sub>3</sub>, R<sub>2</sub>=HR<sub>3</sub>= O-(CH<sub>2</sub>)<sub>2</sub>-O-CH<sub>3</sub>R<sub>1</sub>= O-CH<sub>3</sub>, R<sub>2</sub>=H,R<sub>3</sub>= O-(CH<sub>2</sub>)<sub>2</sub>-O-CH<sub>3</sub>\*

\* This modification can increase or decrease the relative T<sub>m</sub> dependent on the number of modifications and their position (motiff dependant). Substitution at R<sub>1</sub> can be stabilizing, substitution at R<sub>2</sub> is generally greatly destabilizing (unable to form anti conformation), motiffs with stabilizing 5 and 2'-substituent groups are generally additive e.g. increase stability.

Substitution of the O4 and O2 positions of 2'-O-methyl uridine was greatly duplex destabilizing as these modifications remove hydrogen binding sites that would be an expected result. 6-Aza T also showed extreme destabilization as this substitution reduces the pK<sub>a</sub> and shifts the nucleoside toward the enol tautomer resulting in reduced hydrogen bonding.

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Table VI

**DNA strand having at least one modified phosphorus containing internucleoside linkage and the effect on the T<sub>m</sub> against an RNA**

5 **complement:**

	$\Delta T_m/mod +$	$\Delta T_m/mod -$
		phosphorothioate <sup>1</sup>
		phosphoramidate <sup>1</sup>
10		methyl phosphonates <sup>1</sup>
		( <sup>1</sup> one of the non-bridging oxygen atoms replaced with S, N(H)R or -CH <sub>3</sub> )
	phosphoramidate (the 3'-bridging atom replaced with an N(H)R	
15	group, stabilization effect enhanced when also have 2'-F)	

Table VII

**DNA strand having at least one non-phosphorus containing internucleoside linkage and the effect on the T<sub>m</sub> against an RNA**

20 **complement:**

	<b>Positive <math>\Delta T_m/mod</math></b>
25	-CH <sub>2</sub> C(=O)NHCH <sub>2</sub> -*
	-CH <sub>2</sub> C(=O)N(CH <sub>3</sub> )CH <sub>2</sub> -*
	-CH <sub>2</sub> C(=O)N(CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> )CH <sub>2</sub> -*
	-CH <sub>2</sub> C(=O)N(H)CH <sub>2</sub> - (motiff with 5'-propyne on T's)
	-CH <sub>2</sub> N(H)C(=O)CH <sub>2</sub> -*
30	-CH <sub>2</sub> N(CH <sub>3</sub> )OCH <sub>2</sub> -*
	-CH <sub>2</sub> N(CH <sub>3</sub> )N(CH <sub>3</sub> )CH <sub>2</sub> -*

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\* This modification can increase the T<sub>m</sub> of oligonucleotides but can also decrease the T<sub>m</sub> depending on positioning and number (motif dependent).

Notes: In general carbon chain internucleotide linkages were destabilizing to duplex formation. This destabilization was not as severe when double and triple bonds were utilized. The use of glycol and flexible ether linkages were also destabilizing.

Preferred ring structures of the invention for inclusion as a 2'-O modification include cyclohexyl, cyclopentyl and phenyl rings as well as heterocyclic rings having spacial footprints similar to cyclohexyl, cyclopentyl and phenyl rings. Particularly preferred 2'-O-substituent groups of the invention are listed below including an abbreviation for each:

2'-O-(trans 2-methoxy cyclohexyl)	-- 2'-O-(TMCHL)
2'-O-(trans 2-methoxy cyclopentyl)	-- 2'-O-(TMCPL)
2'-O-(trans 2-ureido cyclohexyl)	-- 2'-O-(TUCHL)
2'-O-(trans 2-methoxyphenyl)	-- 2'-O-(2MP)

Structural details for duplexes incorporating such 2'-O-substituents were analyzed using the described AMBER force field program on the Indigo2 SGI machine. The simulated structure maintained a stable A-form geometry throughout the duration of the simulation. The presence of the 2' substitutions locked the sugars in the C3'-endo conformation.

The simulation for the TMCHL modification revealed that the 2'-O-(TMCHL) side chains have a direct interaction with water molecules solvating the duplex. The oxygen atoms in the 2'-O-(TMCHL) side chain are capable of forming a water-mediated interaction with the 3' oxygen of the phosphate backbone. The presence of the two oxygen atoms in the 2'-O-(TMCHL) side chain gives rise to favorable gauche interactions. The barrier for rotation around the O-C-C-O torsion is made even larger by this novel modification. The preferential preorganization in an A-type geometry increases the binding affinity of the 2'-O-(TMCHL) to the target RNA. The locked side chain conformation in the 2'-O-(TMCHL) group created a more favorable pocket

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for binding water molecules. The presence of these water molecules played a key role in holding the side chains in the preferable gauche conformation. While not wishing to be bound by theory, the bulk of the substituent, the diequatorial orientation of the substituents in the cyclohexane ring, the water of hydration and the potential for trapping of metal ions in the conformation generated will additionally contribute to improved binding affinity and nuclease resistance of oligonucleotides incorporating nucleosides having this 2'-O-modification.

As described for the TMCHL modification above, identical computer simulations of the 2'-O-(TMCPL), the 2'-O-(2MP) and 2'-O-(TUCHL) modified oligonucleotides in aqueous solution also illustrate that stable A-form geometry will be maintained throughout the duration of the simulation. The presence of the 2' substitution will lock the sugars in the C3'-endo conformation and the side chains will have direct interaction with water molecules solvating the duplex. The oxygen atoms in the respective side chains are capable of forming a water-mediated interaction with the 3' oxygen of the phosphate backbone. The presence of the two oxygen atoms in the respective side chains give rise to the favorable gauche interactions. The barrier for rotation around the respective O-C-C-O torsions will be made even larger by respective modification. The preferential preorganization in A-type geometry will increase the binding affinity of the respective 2'-O-modified oligonucleotides to the target RNA. The locked side chain conformation in the respective modifications will create a more favorable pocket for binding water molecules. The presence of these water molecules plays a key role in holding the side chains in the preferable gauche conformation. The bulk of the substituent, the diequatorial orientation of the substituents in their respective rings, the water of hydration and the potential trapping of metal ions in the conformation generated will all contribute to improved binding affinity and nuclease resistance of oligonucleotides incorporating nucleosides having these respective 2'-O-modification.

Ribose conformations in C2'-modified nucleosides containing S-methyl groups were examined. To understand the influence of 2'-O-methyl and 2'-S-methyl groups on the conformation of nucleosides, we evaluated the relative



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energies of the 2'-O- and 2'-S-methylguanosine, along with normal deoxyguanosine and riboguanosine, starting from both C2'-endo and C3'-endo conformations using *ab initio* quantum mechanical calculations. All the structures were fully optimized at HF/6-31G\* level and single point energies with electron-correlation were obtained at the MP2/6-31G\*//HF/6-31G\* level. As shown in Table VIII, the C2'-endo conformation of deoxyguanosine is estimated to be 0.6 kcal/mol more stable than the C3'-endo conformation in the gas-phase. The conformational preference of the C2'-endo over the C3'-endo conformation appears to be less dependent upon electron correlation as revealed by the MP2/6-31G\*//HF/6-31G\* values which also predict the same difference in energy. The opposite trend is noted for riboguanosine. At the HF/6-31G\* and MP2/6-31G\*//HF/6-31G\* levels, the C3'-endo form of riboguanosine is shown to be about 0.65 and 1.41 kcal/mol more stable than the C2'endo form, respectively.

15

**Table VIII**

Relative energies\* of the C3'-endo and C2'-endo conformations of representative nucleosides.

	HF/6-31G	MP2/6-31-G	CONTINUUM	AMBER MODEL
dG	0.60	0.56	0.88	0.65
rG	-0.65	-1.41	-0.28	-2.09
2'-O-MeG	-0.89	-1.79	-0.36	-0.86
2'-S-MeG	2.55	1.41	3.16	2.43

25

\* energies are in kcal/mol relative to the C2'-endo conformation

Table VIII also includes the relative energies of 2'-O-methylguanosine and 2'-S-methylguanosine in C2'-endo and C3'-endo conformation. This data indicates the electronic nature of C2'-substitution has a significant impact on the relative stability of these conformations. Substitution of the 2'-O-methyl group increases

30

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the preference for the C3'-endo conformation (when compared to riboguanosine) by about 0.4 kcal/mol at both the HF/6-31G\* and MP2/6-31G\*//HF/6-31G\* levels. In contrast, the 2'-S-methyl group reverses the trend. The C2'-endo conformation is favored by about 2.6 kcal/mol at the HF/6-31G\* level, while the same difference is reduced to 1.41 kcal/mol at the MP2/6-31G\*//HF/6-31G\* level. For comparison, and also to evaluate the accuracy of the molecular mechanical force-field parameters used for the 2'-O-methyl and 2'-S-methyl substituted nucleosides, we have calculated the gas phase energies of the nucleosides. The results reported in Table VIII indicate that the calculated relative energies of these nucleosides compare qualitatively well with the *ab initio* calculations.

Additional calculations were also performed to gauge the effect of solvation on the relative stability of nucleoside conformations. The estimated solvation effect using HF/6-31G\* geometries confirms that the relative energetic preference of the four nucleosides in the gas-phase is maintained in the aqueous phase as well (Table VIII). Solvation effects were also examined using molecular dynamics simulations of the nucleosides in explicit water. From these trajectories, one can observe the predominance of C2'-endo conformation for deoxyriboguanosine and 2'-S-methylriboguanosine while riboguanosine and 2'-O-methylriboguanosine prefer the C3'-endo conformation. These results are in much accord with the available NMR results on 2'-S-methylribonucleosides. NMR studies of sugar puckering equilibrium using vicinal spin-coupling constants have indicated that the conformation of the sugar ring in 2'-S-methylpyrimidine nucleosides show an average of >75% S-character, whereas the corresponding purine analogs exhibit an average of >90% S-pucker [Fraser, A., Wheeler, P., Cook, P.D. and Sanghvi, Y.S., *J. Heterocycl. Chem.*, 1993, 30, 1277-1287]. It was observed that the 2'-S-methyl substitution in deoxynucleoside confers more conformational rigidity to the sugar conformation when compared with deoxyribonucleosides.

Structural features of DNA:RNA, OMe-DNA:RNA and SMe-DNA:RNA hybrids were also observed. The average RMS deviation of the DNA:RNA structure from the starting hybrid coordinates indicate the structure is stabilized

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over the length of the simulation with an approximate average RMS deviation of 1.0 Å. This deviation is due, in part, to inherent differences in averaged structures (i.e. the starting conformation) and structures at thermal equilibrium. The changes in sugar pucker conformation for three of the central base pairs of this hybrid are  
5 in good agreement with the observations made in previous NMR studies. The sugars in the RNA strand maintain very stable geometries in the C3'-endo conformation with ring pucker values near 0°. In contrast, the sugars of the DNA strand show significant variability.

The average RMS deviation of the OMe-DNA:RNA is approximately 1.2  
10 Å from the starting A-form conformation; while the SMe-DNA:RNA shows a slightly higher deviation (approximately 1.8 Å) from the starting hybrid conformation. The SMe-DNA strand also shows a greater variance in RMS deviation, suggesting the S-methyl group may induce some structural fluctuations. The sugar puckers of the RNA complements maintain C3'-endo puckering  
15 throughout the simulation. As expected from the nucleoside calculations, however, significant differences are noted in the puckering of the OMe-DNA and SMe-DNA strands, with the former adopting C3'-endo, and the latter, C1'-exo/C2'-endo conformations.

An analysis of the helicoidal parameters for all three hybrid structures has  
20 also been performed to further characterize the duplex conformation. Three of the more important axis-basepair parameters that distinguish the different forms of the duplexes, X-displacement, propeller twist, and inclination, are reported in Table IX. Usually, an X-displacement near zero represents a B-form duplex; while a negative displacement, which is a direct measure of deviation of the helix from the  
25 helical axis, makes the structure appear more A-like in conformation. In A-form duplexes, these values typically vary from -4Å to -5Å. In comparing these values for all three hybrids, the SMe-DNA:RNA hybrid shows the most deviation from the A-form value, the OMe-DNA:RNA shows the least, and the DNA:RNA is intermediate. A similar trend is also evident when comparing the inclination and  
30 propeller twist values with ideal A-form parameters. These results are further supported by an analysis of the backbone and glycosidic torsion angles of the

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hybrid structures. Glycosidic angles ( $\chi$ ) of A-form geometries, for example, are typically near  $-159^\circ$  while B form values are near  $-102^\circ$ . These angles are found to be  $-162^\circ$ ,  $-133^\circ$ , and  $-108^\circ$  for the OMe-DNA, DNA, and SMe-DNA strands, respectively. All RNA complements adopt an  $\chi$  angle close to  $-160^\circ$ . In addition,

5 “crankshaft” transitions were also noted in the backbone torsions of the central UpU steps of the RNA strand in the SMe-DNA:RNA and DNA:RNA hybrids. Such transitions suggest some local conformational changes may occur to relieve a less favorable global conformation. Taken overall, the results indicate the amount of A-character decreases as OMe-DNA:RNA>DNA:RNA>SMe-

10 DNA:RNA, with the latter two adopting more intermediate conformations when compared to A- and B-form geometries.

**Table IX**

**Average helical parameters derived from**  
**the last 500 ps of simulation time.**

(canonical A-and B-form values are given for comparison)

Helicoidal Parameter	B-DNA (x-ray)	B-DNA (fibre)	A-DNA (fibre)	DNA:RNA	OMe- DNA:RNA	SMe- DNA:RNA
X-disp	1.2	0.0	-5.3	-4.5	-5.4	-3.5
Inclination	-2.3	1.5	20.7	11.6	15.1	0.7
Propeller	-16.4	-13.3	-7.5	-12.7	-15.8	-10.3

The stability of C2'-modified DNA:RNA hybrids was determined. Although the overall stability of the DNA:RNA hybrids depends on several

20 factors including sequence-dependencies and the purine content in the DNA or RNA strands DNA:RNA hybrids are usually less stable than RNA:RNA duplexes and, in some cases, even less stable than DNA:DNA duplexes. Available experimental data attributes the relatively lowered stability of DNA:RNA hybrids largely to its intermediate conformational nature between DNA:DNA (B-family)

25 and RNA:RNA (A-family) duplexes. The overall thermodynamic stability of nucleic acid duplexes may originate from several factors including the

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conformation of backbone, base-pairing and stacking interactions. While it is difficult to ascertain the individual thermodynamic contributions to the overall stabilization of the duplex, it is reasonable to argue that the major factors that promote increased stability of hybrid duplexes are better stacking interactions (electrostatic  $\pi$ - $\pi$ -interactions) and more favorable groove dimensions for hydration. The C2'-S-methyl substitution has been shown to destabilize the hybrid duplex. The notable differences in the rise values among the three hybrids may offer some explanation. While the 2'-S-methyl group has a strong influence on decreasing the base-stacking through high rise values ( $\sim 3.2$  Å), the 2'-O-methyl group makes the overall structure more compact with a rise value that is equal to that of A-form duplexes ( $\sim 2.6$  Å). Despite its overall A-like structural features, the SMe-DNA:RNA hybrid structure possesses an average rise value of 3.2 Å which is quite close to that of B-family duplexes. In fact, some local base-steps (CG steps) may be observed to have unusually high rise values (as high as 4.5 Å). Thus, the greater destabilization of 2'-S-methyl substituted DNA:RNA hybrids may be partly attributed to poor stacking interactions.

**Table X****Minor groove widths averaged****over the last 500 ps of simulation time**

Phosphate Distance	DNA:RNA	OMe-DNA:RNA	SMe-DNA:RNA	DNA:RNA (B-form)	RNA:RNA (A-form)
P5-P20	15.27	16.82	13.73	14.19	17.32
P6-P19	15.52	16.79	15.73	12.66	17.12
P7-P18	15.19	16.40	14.08	11.10	16.60
P8-P17	15.07	16.12	14.00	10.98	16.14
P9-P16	15.29	16.25	14.98	11.65	16.93
P10-P15	15.37	16.57	13.92	14.05	17.69

In addition to the modifications described above, the nucleotides of the

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oligonucleotides of the invention can have a variety of other modification so long as these other modifications enhance one or more of the desired properties described above. Thus, for nucleotides that are incorporated into oligonucleotides of the invention, these nucleotides can have sugar portions that correspond to  
5 naturally-occurring sugars or modified sugars. Representative modified sugars include carbocyclic or acyclic sugars, sugars having substituent groups at their 2' position, sugars having substituent groups at their 3' position, and sugars having substituents in place of one or more hydrogen atoms of the sugar. Other altered base moieties and altered sugar moieties are disclosed in United States Patent  
10 3,687,808 and PCT application PCT/US89/02323.

Altered base moieties or altered sugar moieties also include other modifications consistent with the spirit of this invention. Such oligonucleotides are best described as being structurally distinguishable from, yet functionally interchangeable with, naturally occurring or synthetic wild type oligonucleotides.  
15 All such oligonucleotides are comprehended by this invention so long as they function effectively to mimic the structure of a desired RNA or DNA strand. A class of representative base modifications include tricyclic cytosine analog, termed "G clamp" (Lin, *et al.*, *J. Am. Chem. Soc.* 1998, 120, 8531). This analog makes four hydrogen bonds to a complementary guanine (G) within a helix by  
20 simultaneously recognizing the Watson-Crick and Hoogsteen faces of the targeted G. This G clamp modification when incorporated into phosphorothioate oligonucleotides, dramatically enhances antisense potencies in cell culture. The oligonucleotides of the invention also can include phenoxazine-substituted bases of the type disclosed by Flanagan, *et al.*, *Nat. Biotechnol.* 1999, 17(1), 48-52.

25 It is preferred to target specific nucleic acids for RNAi methodologies. "Targeting" an RNAi compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (usually a mRNA transcribed from the gene)  
30 whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. Within the context of the present

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invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule),  
5 the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon  
10 sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions.  
15 In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of the target, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and  
20 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and  
25 "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the  
30 translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art

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to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the



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capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

RNAi and other compounds of the invention which hybridize to the target and inhibit expression of the target are identified through experimentation, and the sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The target sites to which these preferred sequences are complementary are hereinbelow referred to as "active sites" and are therefore preferred sites for targeting. Therefore another embodiment of the invention encompasses compounds, including primers, probes, siRNAs, other double stranded RNAs including RNAi or gene silencing agents, ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to these active sites.

Some representative siRNA oligomers as per the invention include:

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	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, all PO
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, all PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-thiophosphate, 3'-OH, all PS
5	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, all F/ PO
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, all F/PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , all PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , all PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , all PS
10	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> / all PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-thiophosphate, 3'-OH, <u>F</u> / all PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , all PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , all PS
15	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , all PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , all PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , all PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , <u>PO</u> , rest PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>OMe</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>OMe</u> , PS
20	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>OMe</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>OMe</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>OMe</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>OMe</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>OMe</u> , PS
25	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , <u>OMe</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , <u>OMe</u> , PS
	5'-CCU UUU UGU CUC UGG CC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , <u>OMe</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , <u>OMe</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , <u>PO</u> , <u>OMe</u> , rest PS
30	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , <u>PO</u> , <u>OMe</u> , rest PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , <u>deoxy</u> , PS
35	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , <u>deoxy</u> , PO
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>LNA</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>LNA</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>LNA</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>LNA</u> , PS
40	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>LNA</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>LNA</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , <u>LNA</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , <u>LNA</u> , PS
45	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , <u>LNA</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>F</u> , <u>LNA</u> , PS
	5'-CCU UUU UGU CUC UGG UCC UU-3'	5'-phosphate, 3'-OH, <u>LNA/PO</u> , PS

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5'-CCU UUU UGU CUC UGG UCC UU-3'  
 5'-CCU UUU UGU CUC UGG UCC UU-3'  
 5'-CCU UUU UGU CUC UGG UCC UU-3'

5'-phosphate, 3'-OH, LNA/PO, PS  
 5'-phosphate, 3'-OH, LNA/PO, PS  
 5'-phosphate, 3'-OH, LNA/PO, PS

5 Definitions:

	PS =	phosphorothioate internucleotide linkage
	PO =	phosphodiester internucleotide linkage
	<u>PO</u> =	phosphodiester internucleotide linkage
	<u>deoxy</u> =	2'-deoxy nucleotide
10	<u>F</u> =	2'-fluoro
	3'-OH =	3'-terminus has a hydroxyl group
	OMe =	2'-O-methyl
	LNA =	locked nucleic acid
	LNA/PO =	LNA with phosphodiester internucleotide linkages
15	5'-phosphate =	5'-terminus has a phosphate group

Note: Each nucleoside marked to indicate a specific linkage type indicates that the particular linkage (e.g. PO or PS) is attached to the 5'-position of that nucleoside completing the internucleoside linkage by making a second attachment to an adjacent nucleoside at its 3'-position.

20

Oligonucleotide compounds of the invention can be used as research reagents and diagnostics. For example, siRNAs, which are able to inhibit gene expression with exquisite specificity, can be used by those of ordinary skill to elucidate the function of particular genes. SiRNA compounds may also be used, for example, to distinguish between functions of various members of a biological pathway. RNAi modulation is being used for target validation with respect to selected gene targets and as such is useful as a research tool.

In the context of this invention, the term "modified oligonucleotide" refers to a polymeric structure capable of hybridizing a region of a nucleic acid molecule. This term includes oligonucleotides, oligonucleosides, oligonucleotide analogs, oligonucleotide mimetics and combinations of these. Modified oligonucleotides can be prepared to be linear or circular and may include branching. They can be prepared single stranded or double stranded and may include overhangs. In general a modified oligonucleotide comprises a backbone of linked monomeric subunits where each linked monomeric subunit is directly or indirectly attached to a heterocyclic base moiety. The linkages joining the monomeric subunits, the sugar moieties or surrogates and the heterocyclic base moieties can be independently modified giving rise to a plurality of motifs for the

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resulting modified oligonucleotides including hemimers, gapmers and chimeras.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base moiety. The two most common classes of such heterocyclic bases are purines and pyrimidines.

5 Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric  
10 compound. The respective ends of this linear polymeric structure can be joined to form a circular structure by hybridization or by formation of a covalent bond, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside linkages of the oligonucleotide. The normal  
15 internucleoside linkage of RNA and DNA is a 3' to 5' phosphodiester linkage.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside linkages. The term "oligonucleotide analog"  
20 refers to oligonucleotides that have one or more non-naturally occurring portions which function in a similar manner to oligonucleotides. Such non-naturally occurring oligonucleotides are often preferred to the naturally occurring forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of  
25 nucleases.

In the context of this invention, the term "oligonucleoside" refers to nucleosides that are joined by internucleoside linkages that do not have phosphorus atoms. Internucleoside linkages of this type include short chain alkyl, cycloalkyl, mixed heteroatom alkyl, mixed heteroatom cycloalkyl, one or more  
30 short chain heteroatomic and one or more short chain heterocyclic. These internucleoside linkages include but are not limited to siloxane, sulfide, sulfoxide,

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sulfone, acetyl, formacetyl, thioformacetyl, methylene formacetyl, thioformacetyl, alkeneyl, sulfamate; methyleneimino, methylenehydrazino, sulfonate, sulfonamide, amide and others having mixed N, O, S and CH<sub>2</sub> component parts.

Representative United States patents that teach the preparation of the  
5 above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315;  
5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938;  
5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086;  
5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070;  
5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of  
10 which are commonly owned with this application, and each of which is herein  
incorporated by reference.

In the context of this invention, the term "oligonucleotide mimetic" refers  
to an oligonucleotide wherein the backbone of the nucleotide units has been  
replaced with novel groups. Although the term is intended to include modified  
15 oligonucleotides wherein only the furanose ring or both the furanose ring and the  
internucleotide linkage are replaced with novel groups, replacement of only the  
furanose ring is also referred to in the art as being a sugar surrogate.

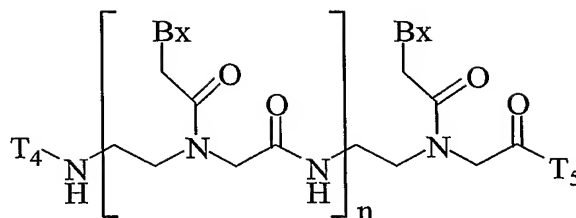
Oligonucleotide mimetics can be further modified to incorporate one or more  
modified heterocyclic base moieties to enhance properties such as hybridization.

20 One oligonucleotide mimetic that has been reported to have excellent  
hybridization properties, is peptide nucleic acids (PNA). The backbone in PNA  
compounds is two or more linked aminoethylglycine units which gives PNA an  
amide containing backbone. The heterocyclic base moieties are bound directly or  
indirectly to aza nitrogen atoms of the amide portion of the backbone.

25 Representative United States patents that teach the preparation of PNA  
compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and  
5,719,262, each of which is herein incorporated by reference. Further teaching of  
PNA compounds can be found in Nielsen et al., Science, 1991, 254, 1497-1500.

PNA has been modified to incorporate numerous modifications since the  
30 basic PNA structure was first prepared. The basic structure is shown below:

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wherein

Bx is a heterocyclic base moiety;

T<sub>4</sub> is hydrogen, an amino protecting group, -C(O)R<sub>5</sub>, substituted or  
 5 unsubstituted C<sub>1</sub>-C<sub>10</sub> alkyl, substituted or unsubstituted C<sub>2</sub>-C<sub>10</sub> alkenyl, substituted  
 or unsubstituted C<sub>2</sub>-C<sub>10</sub> alkynyl, alkylsulfonyl, arylsulfonyl, a chemical functional  
 group, a reporter group, a conjugate group, a D or L α-amino acid linked via the  
 α-carboxyl group or optionally through the ω-carboxyl group when the amino  
 acid is aspartic acid or glutamic acid or a peptide derived from D, L or mixed D  
 10 and L amino acids linked through a carboxyl group, wherein the substituent  
 groups are selected from hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro,  
 thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl;

T<sub>5</sub> is -OH, -N(Z<sub>1</sub>)Z<sub>2</sub>, R<sub>5</sub>, D or L α-amino acid linked via the α-amino  
 group or optionally through the ω-amino group when the amino acid is lysine or  
 15 ornithine or a peptide derived from D, L or mixed D and L amino acids linked  
 through an amino group, a chemical functional group, a reporter group or a  
 conjugate group;

Z<sub>1</sub> is hydrogen, C<sub>1</sub>-C<sub>6</sub> alkyl, or an amino protecting group;

Z<sub>2</sub> is hydrogen, C<sub>1</sub>-C<sub>6</sub> alkyl, an amino protecting group, -C(=O)-(CH<sub>2</sub>)<sub>n</sub>-J-  
 20 Z<sub>3</sub>, a D or L α-amino acid linked via the α-carboxyl group or optionally through  
 the ω-carboxyl group when the amino acid is aspartic acid or glutamic acid or a  
 peptide derived from D, L or mixed D and L amino acids linked through a  
 carboxyl group;

Z<sub>3</sub> is hydrogen, an amino protecting group, -C<sub>1</sub>-C<sub>6</sub> alkyl, -C(=O)-CH<sub>3</sub>,  
 25 benzyl, benzoyl, or -(CH<sub>2</sub>)<sub>n</sub>-N(H)Z<sub>1</sub>;

each J is O, S or NH;

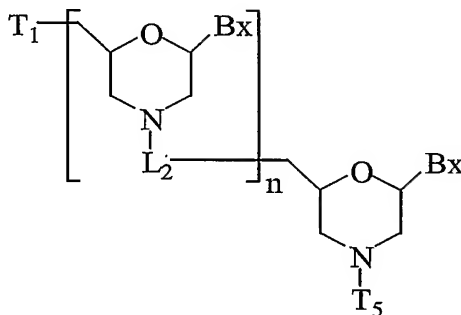
R<sub>5</sub> is a carbonyl protecting group; and

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n is from 2 to about 50.

Another class of oligonucleotide mimetic that has been studied is based on linked morpholino units (morpholino nucleic acid) having heterocyclic bases attached to the morpholino ring. A number of linking groups have been reported that link the morpholino monomeric units in a morpholino nucleic acid. A preferred class of linking groups have been selected to give a non-ionic modified oligonucleotide. The non-ionic morpholino-based modified oligonucleotides are less likely to have undesired interactions with cellular proteins. Morpholino-based modified oligonucleotides are non-ionic mimics of oligonucleotides which are less likely to form undesired interactions with cellular proteins (Dwayne A. Braasch and David R. Corey, *Biochemistry*, **2002**, *41*(14), 4503-4510). Morpholino-based modified oligonucleotides are disclosed in United States Patent 5,034,506, issued July 23, 1991. The morpholino class of modified oligonucleotides have been prepared having a variety of different linking groups joining the monomeric subunits.

Morpholino nucleic acids have been prepared having a variety of different linking groups ( $L_2$ ) joining the monomeric subunits. The basic formula is shown below:



wherein

$T_1$  is hydroxyl or a protected hydroxyl;

$T_5$  is hydrogen or a phosphate or phosphate derivative;

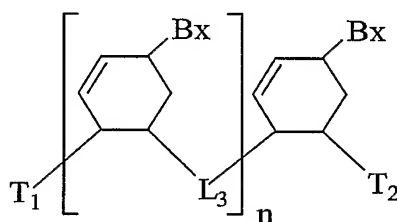
$L_2$  is a linking group; and

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n is from 2 to about 50.

A further class of oligonucleotide mimetic is referred to as cyclohexenyl nucleic acids (CeNA). The furanose ring normally present in an DNA/RNA molecule is replaced with a cyclohexenyl ring. CeNA DMT protected  
 5 phosphoramidite monomers have been prepared and used for modified oligonucleotide synthesis following classical phosphoramidite chemistry. Fully modified CeNA modified oligonucleotides and oligonucleotides having specific positions modified with CeNA have been prepared and studied (see Wang *et al.*, *J. Am. Chem. Soc.*, **2000**, 122, 8595-8602). In general the incorporation of CeNA  
 10 monomers into a DNA chain increases its stability of a DNA/RNA hybrid. CeNA oligoadenylates formed complexes with RNA and DNA complements with similar stability to the native complexes. The study of incorporating CeNA structures into natural nucleic acid structures was shown by NMR and circular dichroism to proceed with easy conformational adaptation. Furthermore the incorporation of  
 15 CeNA into a sequence targeting RNA was stable to serum and able to activate E. Coli RNase resulting in cleavage of the target RNA strand.

The general formula of CeNA is shown below:



20

wherein

each Bx is a heterocyclic base moiety;

T<sub>1</sub> is hydroxyl or a protected hydroxyl; and

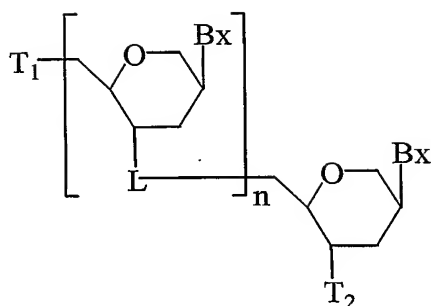
T<sub>2</sub> is hydroxyl or a protected hydroxyl.

25

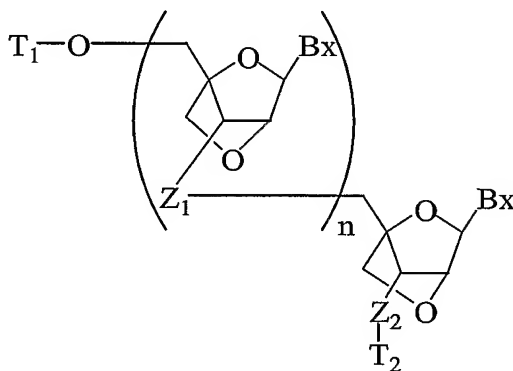
Another class of oligonucleotide mimetic (anhydrohexitol nucleic acid) can be prepared from one or more anhydrohexitol nucleosides (see, Wouters and Herdewijn, *Bioorg. Med. Chem. Lett.*, **1999**, 9, 1563-1566) and would have the general formula:



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A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 4' carbon atom of the sugar ring thereby forming a 2'-C,4'-C-oxymethylene linkage thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene  $(-\text{CH}_2-)_n$  group bridging the 2' oxygen atom and the 4' carbon atom wherein  $n$  is 1 or 2 (Singh et al., Chem. Commun., 1998, 4, 455-456). LNA and LNA analogs display very high duplex thermal stabilities with complementary DNA and RNA ( $T_m = +3$  to  $+10$  C), stability towards 3'-exonucleolytic degradation and good solubility properties. The basic structure of LNA showing the bicyclic ring system is shown below:



The conformations of LNAs determined by 2D NMR spectroscopy have shown that the locked orientation of the LNA nucleotides, both in single-stranded LNA and in duplexes, constrains the phosphate backbone in such a way as to introduce a higher population of the N-type conformation (Petersen et al., J. Mol. Recognit., 2000, 13, 44-53). These conformations are associated with improved

stacking of the nucleobases (Wengel et al., *Nucleosides Nucleotides*, 1999, 18, 1365-1370).

LNA has been shown to form exceedingly stable LNA:LNA duplexes (Koshkin et al., *J. Am. Chem. Soc.*, 1998, 120, 13252-13253). LNA:LNA  
5 hybridization was shown to be the most thermally stable nucleic acid type duplex system, and the RNA-mimicking character of LNA was established at the duplex level. Introduction of 3 LNA monomers (T or A) significantly increased melting points ( $T_m = +15/+11$ ) toward DNA complements. The universality of LNA-mediated hybridization has been stressed by the formation of exceedingly stable  
10 LNA:LNA duplexes. The RNA-mimicking of LNA was reflected with regard to the N-type conformational restriction of the monomers and to the secondary structure of the LNA:RNA duplex.

LNAs also form duplexes with complementary DNA, RNA or LNA with high thermal affinities. Circular dichroism (CD) spectra show that duplexes  
15 involving fully modified LNA (esp. LNA:RNA) structurally resemble an A-form RNA:RNA duplex. Nuclear magnetic resonance (NMR) examination of an LNA:DNA duplex confirmed the 3'-endo conformation of an LNA monomer. Recognition of double-stranded DNA has also been demonstrated suggesting strand invasion by LNA. Studies of mismatched sequences show that LNAs obey  
20 the Watson-Crick base pairing rules with generally improved selectivity compared to the corresponding unmodified reference strands.

Novel types of LNA-modified oligonucleotides, as well as the LNAs, are useful in a wide range of diagnostic and therapeutic applications. Among these are antisense applications, PCR applications, strand-displacement oligomers,  
25 substrates for nucleic acid polymerases and generally as nucleotide based drugs.

Potent and nontoxic antisense oligonucleotides containing LNAs have been described (Wahlestedt et al., *Proc. Natl. Acad. Sci. U. S. A.*, 2000, 97, 5633-5638.) The authors have demonstrated that LNAs confer several desired properties to antisense agents. LNA/DNA copolymers were not degraded readily  
30 in blood serum and cell extracts. LNA/DNA copolymers exhibited potent antisense activity in assay systems as disparate as G-protein-coupled receptor

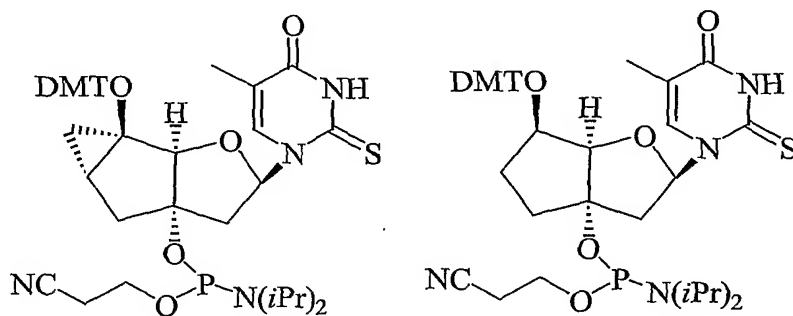
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signaling in living rat brain and detection of reporter genes in *Escherichia coli*. Lipofectin-mediated efficient delivery of LNA into living human breast cancer cells has also been accomplished.

The synthesis and preparation of the LNA monomers adenine, cytosine,  
5 guanine, 5-methyl-cytosine, thymine and uracil, along with their oligomerization, and nucleic acid recognition properties have been described (Koshkin et al., Tetrahedron, 1998, 54, 3607-3630). LNAs and preparation thereof are also described in WO 98/39352 and WO 99/14226.

The first analogs of LNA, phosphorothioate-LNA and 2'-thio-LNAs, have  
10 also been prepared (Kumar et al., Bioorg. Med. Chem. Lett., 1998, 8, 2219-2222). Preparation of locked nucleoside analogs containing oligodeoxyribonucleotide duplexes as substrates for nucleic acid polymerases has also been described (Wengel et al., PCT International Application WO 98-DK393 19980914). Furthermore, synthesis of 2'-amino-LNA, a novel conformationally restricted  
15 high-affinity oligonucleotide analog with a handle has been described in the art (Singh et al., J. Org. Chem., 1998, 63, 10035-10039). In addition, 2'-Amino- and 2'-methylamino-LNA's have been prepared and the thermal stability of their duplexes with complementary RNA and DNA strands has been previously reported.

20 Further oligonucleotide mimetics have been prepared to include bicyclic and tricyclic nucleoside analogs having the formulas (amidite monomers shown):



(see Steffens *et al.*, *Helv. Chim. Acta*, **1997**, 80, 2426-2439; Steffens *et al.*, *J. Am.*  
25 *Chem. Soc.*, **1999**, 121, 3249-3255; and Renneberg *et al.*, *J. Am. Chem. Soc.*,  
**2002**, 124, 5993-6002). These modified nucleoside analogs have been

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oligomerized using the phosphoramidite approach and the resulting modified oligonucleotides containing tricyclic nucleoside analogs have shown increased thermal stabilities ( $T_m$ 's) when hybridized to DNA, RNA and itself. Modified oligonucleotides containing bicyclic nucleoside analogs have shown thermal

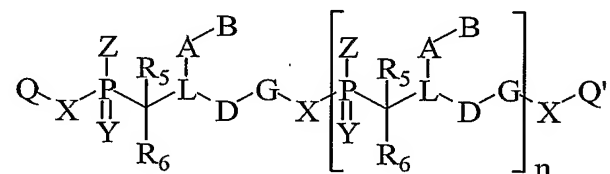
5 stabilities approaching that of DNA duplexes.

Another class of oligonucleotide mimetic is referred to as phosphonomonoester nucleic acids incorporate a phosphorus group in a backbone the backbone. This class of oligonucleotide mimetic is reported to have useful physical and biological and pharmacological properties in the areas of inhibiting

10 gene expression (antisense oligonucleotides, ribozymes, sense oligonucleotides and triplex-forming oligonucleotides), as probes for the detection of nucleic acids and as auxiliaries for use in molecular biology.

The general formula (for definitions of Markush variables see: United States Patents 5,874,553 and 6,127,346 herein incorporated by reference in

15 their entirety) is shown below.



Another oligonucleotide mimetic has been reported wherein the furanosyl ring has been replaced by a cyclobutyl moiety.

20 The internucleotide linkage found in native nucleic acids is a phosphodiester linkage. This linkage has not been the linkage of choice for synthetic oligonucleotides that are for the most part targeted to a portion of a nucleic acid such as mRNA because of stability problems e.g. degradation by nucleases. Preferred internucleotide linkages and internucleoside linkages as is

25 the case for non phosphate ester type linkages include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates,

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phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleoside linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

In the *C. elegans* system, modification of the internucleotide linkage (phosphorothioate) did not significantly interfere with RNAi activity. Based on this observation, it is suggested that the oligomeric compounds of the invention can also have one or more modified internucleoside linkages. A preferred phosphorus containing modified internucleoside linkage is the phosphorothioate internucleoside linkage.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In more preferred embodiments of the invention, modified oligonucleotides have one or more phosphorothioate and/or heteroatom internucleoside linkages, in particular -CH<sub>2</sub>-NH-O-CH<sub>2</sub>-, -CH<sub>2</sub>-N(CH<sub>3</sub>)-O-CH<sub>2</sub>- [known as a methylene (methylimino) or MMI backbone], -CH<sub>2</sub>-O-N(CH<sub>3</sub>)-CH<sub>2</sub>-, -CH<sub>2</sub>-N(CH<sub>3</sub>)-N(CH<sub>3</sub>)-CH<sub>2</sub>- and -O-N(CH<sub>3</sub>)-CH<sub>2</sub>-CH<sub>2</sub>- [wherein the native phosphodiester internucleotide linkage is represented as -O-P(=O)(OH)-O-CH<sub>2</sub>-]. The MMI type internucleoside linkages are disclosed in the above referenced U.S.

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patent 5,489,677. Preferred amide internucleoside linkages are disclosed in the above referenced U.S. patent 5,602,240.

Modified oligonucleotides can have a variety of substituent groups attached at various positions. Furanosyl sugar moieties found in nucleoside units of native nucleic acids as well as a wide range of modified nucleoside units of modified oligonucleotides can be substituted at a number of positions. The most frequently substituted position is the 2'-position (ribose and arabinose). The 3', 4', and 5' have also been substituted with groups generally referred to as sugar substituent groups. Preferred sugar substituent groups include: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C<sub>1</sub> to C<sub>10</sub> alkyl or C<sub>2</sub> to C<sub>10</sub> alkenyl and alkynyl. Particularly preferred are O[(CH<sub>2</sub>)<sub>n</sub>O]<sub>m</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>OCH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>NH<sub>2</sub>, O(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>ONH<sub>2</sub>, and O(CH<sub>2</sub>)<sub>n</sub>ON[(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>]<sub>2</sub>, where n and m are from 1 to about 10. Other sugar substituent groups include: C<sub>1</sub> to C<sub>10</sub> lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, SOCH<sub>3</sub>, SO<sub>2</sub>CH<sub>3</sub>, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties.

More preferred sugar substituent groups that are more frequently covalently attached to the 2'-sugar position include methoxyethoxy (-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as -O-(2-methoxyethyl) or MOE) (Martin et al., *Helv. Chim. Acta*, **1995**, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred 2'-modification includes dimethylaminoethoxy, i.e., a -O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub> group, also known as DMAOE, as described in examples hereinbelow, and -dimethylaminoethoxyethoxy (also known in the art as -O-dimethylaminoethoxyethyl or -DMAEOE), i.e., O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>2</sub>)<sub>2</sub>, also described in examples hereinbelow.

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Other preferred sugar substituent groups that are more frequently covalently attached to the 2'-sugar position include methoxy (-O-CH<sub>3</sub>), aminopropoxy (-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>), allyl (-CH<sub>2</sub>-CH=CH<sub>2</sub>), -O-allyl (-O-CH<sub>2</sub>-CH=CH<sub>2</sub>) and fluoro (-F). A 2'-substituent group on a furanosyl ring can be in the  
5 ribo (down) or arabino (up) position. Preferred 2'-arabino modifications include fluoro and hydroxy. Similar modifications may also be made at other positions on a modified oligonucleotide, particularly the 3' position of the sugar for a 2'-5' linked modified oligonucleotide, the 3'-terminus and the 5'-position of the 5'-terminus.

10 Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of  
15 which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Modified oligonucleotides may also include nucleobase (often referred to in the art simply as "base" or "heterocyclic base moiety") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the  
20 purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases also referred herein as heterocyclic base moieties include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-  
25 propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH<sub>3</sub>) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and  
30 guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-

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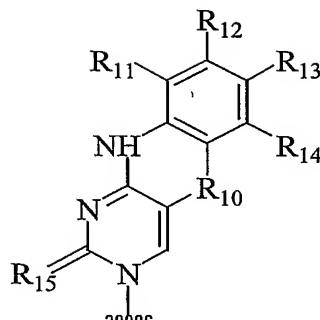
amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

Heterocyclic base moieties may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the modified oligonucleotides of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

In one aspect of the present invention modified oligonucleotides are prepared having polycyclic heterocyclic compounds in place of one or more heterocyclic base moieties. A number of tricyclic heterocyclic compounds have been previously reported. These compounds are routinely used in antisense applications to increase the binding properties of the modified strand to a target strand. The most studied modifications are targeted to guanosines hence they have been termed G-clamps or cytidine analogs. Many of these polycyclic heterocyclic compounds have the general formula:



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Representative cytosine analogs that make 3 hydrogen bonds with a guanosine in a second strand include 1,3-diazaphenoxazine-2-one ( $R_{10} = O$ ,  $R_{11} - R_{14} = H$ ) [Kurchavov, *et al.*, *Nucleosides and Nucleotides*, 1997, 16, 1837-1846],

5 1,3-diazaphenothiazine-2-one ( $R_{10} = S$ ,  $R_{11} - R_{14} = H$ ), [Lin, K.-Y.; Jones, R. J.; Matteucci, M. J. *Am. Chem. Soc.* 1995, 117, 3873-3874] and 6,7,8,9-tetrafluoro-1,3-diazaphenoxazine-2-one ( $R_{10} = O$ ,  $R_{11} - R_{14} = F$ ) [Wang, J.; Lin, K.-Y., Matteucci, M. *Tetrahedron Lett.* 1998, 39, 8385-8388]. Incorporated into

10 oligonucleotides these base modifications were shown to hybridize with complementary guanine and the latter was also shown to hybridize with adenine and to enhance helical thermal stability by extended stacking interactions (also see U.S. Patent Application entitled "Modified Peptide Nucleic Acids" filed May 24, 2002, Serial number 10/155,920; and U.S. Patent Application entitled "Nuclease

15 Resistant Chimeric Oligonucleotides" filed May 24, 2002, Serial number 10/013,295, both of which are commonly owned with this application and are herein incorporated by reference in their entirety).

Further helix-stabilizing properties have been observed when a cytosine analog/substitute has an aminoethoxy moiety attached to the rigid 1,3-diazaphenoxazine-2-one scaffold ( $R_{10} = O$ ,  $R_{11} = -O-(CH_2)_2-NH_2$ ,  $R_{12-14} = H$ ) [Lin, K.-Y.; Matteucci, M. J. *Am. Chem. Soc.* 1998, 120, 8531-8532]. Binding studies demonstrated that a single incorporation could enhance the binding affinity of a model oligonucleotide to its complementary target DNA or RNA with a  $\Delta T_m$  of up to  $18^\circ$  relative to 5-methyl cytosine ( $dC5^{me}$ ), which is the highest known affinity

25 enhancement for a single modification, yet. On the other hand, the gain in helical

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stability does not compromise the specificity of the oligonucleotides. The  $T_m$  data indicate an even greater discrimination between the perfect match and mismatched sequences compared to dC5<sup>me</sup>. It was suggested that the tethered amino group serves as an additional hydrogen bond donor to interact with the Hoogsteen face, namely the O6, of a complementary guanine thereby forming 4 hydrogen bonds. This means that the increased affinity of G-clamp is mediated by the combination of extended base stacking and additional specific hydrogen bonding.

Further tricyclic heterocyclic compounds and methods of using them that are amenable to the present invention are disclosed in United States Patent Serial Number 6,028,183, which issued on May 22, 2000, and United States Patent Serial Number 6,007,992, which issued on December 28, 1999, the contents of both are commonly assigned with this application and are incorporated herein in their entirety.

The enhanced binding affinity of the phenoxazine derivatives together with their uncompromised sequence specificity makes them valuable nucleobase analogs for the development of more potent antisense-based drugs. In fact, promising data have been derived from in vitro experiments demonstrating that heptanucleotides containing phenoxazine substitutions are capable to activate RNaseH, enhance cellular uptake and exhibit an increased antisense activity [Lin, K.-Y.; Matteucci, M. J. Am. Chem. Soc. 1998, 120, 8531-8532]. The activity enhancement was even more pronounced in case of G-clamp, as a single substitution was shown to significantly improve the in vitro potency of a 20mer 2'-deoxyphosphorothioate oligonucleotides [Flanagan, W. M.; Wolf, J.J.; Olson, P.; Grant, D.; Lin, K.-Y.; Wagner, R. W.; Matteucci, M. Proc. Natl. Acad. Sci. USA, 1999, 96, 3513-3518]. Nevertheless, to optimize oligonucleotide design and to better understand the impact of these heterocyclic modifications on the biological activity, it is important to evaluate their effect on the nuclease stability of the oligomers.

Further modified polycyclic heterocyclic compounds useful as heterocyclic bases are disclosed in but not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273;

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5,367,066; 5,432,272; 5,434,257; 5,457,187; 5,459,255; 5,484,908; 5,502,177;  
5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985;  
5,646,269; 5,750,692; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, and  
Unites States Patent Application Serial number 09/996,292 filed November 28,  
5 2001, certain of which are commonly owned with the instant application, and each  
of which is herein incorporated by reference.

A further preferred substitution that can be appended to the modified  
oligonucleotides of the invention involves the linkage of one or more moieties or  
conjugates which enhance the activity, cellular distribution or cellular uptake of  
10 the resulting modified oligonucleotides. In one embodiment such modified  
modified oligonucleotides are prepared by covalently attaching conjugate groups  
to functional groups such as hydroxyl or amino groups. Conjugate groups of the  
invention include intercalators, reporter molecules, polyamines, polyamides, poly-  
ethylene glycols, polyethers, groups that enhance the pharmacodynamic properties  
15 of oligomers, and groups that enhance the pharmacokinetic properties of  
oligomers. Typical conjugates groups include cholesterol, lipids, phospholipids,  
biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins,  
rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic  
properties, in the context of this invention, include groups that improve oligomer  
20 uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-  
specific hybridization with RNA. Groups that enhance the pharmacokinetic  
properties, in the context of this invention, include groups that improve oligomer  
uptake, distribution, metabolism or excretion. Representative conjugate groups  
are disclosed in International Patent Application PCT/US92/09196, filed October  
25 23, 1992 the entire disclosure of which is incorporated herein by reference.

Conjugate moieties include but are not limited to lipid moieties such as a  
cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, 86, 6553-  
6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Let.*, **1994**, 4, 1053-  
1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*,  
30 **1992**, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Let.*, **1993**, 3, 2765-  
2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, 20, 533-538),

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an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk et al., *Biochimie*, **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-  
5 phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, *14*, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, *1264*, 229-  
10 237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, *277*, 923-937).

The modified oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-pranoprofen, carprofen, dansylsarcosine,  
15 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its  
20 entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077;  
25 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552;  
30 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant

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application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a modified oligonucleotide to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single modified oligonucleotide or even at  
5 a single monomeric subunit such as a nucleoside within a modified oligonucleotide. The present invention also includes modified oligonucleotides which are chimeric compounds. "Chimeric" modified oligonucleotides or "chimeras," in the context of this invention, are modified oligonucleotides which contain two or more chemically distinct regions, each made up of at least one  
10 monomer unit, i.e., a nucleotide in the case of a nucleic acid based oligomer.

Chimeric oligomeric compounds typically contain at least one region modified so as to confer increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the modified oligonucleotide may serve as a substrate for  
15 enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of inhibition of gene expression. Consequently, comparable results can often be obtained with shorter  
20 modified oligonucleotides when chimeras are used, compared to for example phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric modified oligonucleotides of the invention may be formed as  
25 composite structures of two or more oligonucleotides, oligonucleotide analogs, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids hemimers, gapmers or inverted gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830;  
30 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly

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owned with the instant application, and each of which is herein incorporated by reference in its entirety.

The modified oligonucleotide compounds used in accordance with this invention may be conveniently and routinely made through the well-known  
5 technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

10 The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor-targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation  
15 of such uptake, distribution and/or absorption-assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152;  
20 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon  
25 administration to an animal, including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an  
30 inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or

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conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin *et al.*, published December 9, 1993 or in WO 94/26764 and U.S. 5,770,713 to Imbach *et al.*

5           The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or  
10   amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chlorprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge *et al.*, "Pharmaceutical  
15   Salts," *J. of Pharma Sci.*, 1977, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their  
20   respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or  
25   inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or  
30   phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic

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acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an



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animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of a particular target gene is treated by administering compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an  
5 effective amount of a compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the modified oligonucleotide compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The modified oligonucleotide compounds of the invention are useful for  
10 research and diagnostics, because these compounds can be prepared to hybridize to nucleic acids encoding a particular protein, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the modified oligonucleotides of the invention with a nucleic acid encoding a particular protein can be detected by means known in the art. Such means may include conjugation  
15 of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting protein levels in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the modified oligonucleotide compounds of the  
20 invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or  
25 aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to  
30 be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration

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may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred

5 topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative

10 (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to

15 cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C<sub>1-10</sub> alkyl

20 ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or

25 granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitables. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration

30 enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile

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acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucolic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate and sodium glycodihydrofusidate. Preferred fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether.

Oligonucleotides of the invention may be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for oligonucleotides and their preparation are described in detail in United States applications 08/886,829 (filed July 1, 1997), 09/108,673 (filed July 1, 1998),

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09/256,515 (filed February 23, 1999), 09/082,624 (filed May 21, 1998) and 09/315,298 (filed May 20, 1999), each of which is incorporated herein by reference in their entirety.

5       Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

10       Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

15       The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active  
20       ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

25       The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

30       In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams

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include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

#### Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1  $\mu\text{m}$  in diameter (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi *et al.*, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be of either the water-in-oil (w/o) or the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase, the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase, the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the

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case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a  
5 system of oil droplets enclosed in globules of water stabilized in an oily continuous phase provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of  
10 emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants,  
15 naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the  
20 literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The  
25 ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in  
30 *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

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Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride,

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esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation.

Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents  
5 such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and  
10 Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of ease of formulation, as well as efficacy from an absorption and bioavailability standpoint (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245;  
15 Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of  
20 oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically  
25 microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as  
thermodynamically stable, isotropically clear dispersions of two immiscible  
30 liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*,



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Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215).

Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte.

Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprates (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300,

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PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol.

The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols,

5 polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides *et al.*, *Pharmaceutical Research*, **1994**, 11, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, **1993**, 13, 205).

Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of

15 preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides *et al.*, *Pharmaceutical Research*, **1994**, 11, 1385; Ho *et al.*, *J. Pharm. Sci.*, **1996**, 85, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when  
20 formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the  
25 gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and  
30 penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention.

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Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes  
5 has been discussed above.

### Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include  
10 monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

15 Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up  
20 by macrophages *in vivo*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

25 Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988,  
30 Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface

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charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes and as the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al.*, *Biochem. Biophys. Res. Commun.*, **1987**, *147*, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to

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cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, *Journal of Controlled Release*, **1992**, *19*, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (*e.g.* as a solution or as an emulsion) were ineffective (Weiner *et al.*, *Journal of Drug Targeting*, **1992**, *2*, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis *et al.*, *Antiviral Research*, **1992**, *18*, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/ cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu *et al.* *S.T.P. Pharma. Sci.*, **1994**, *4*, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as

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used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G<sub>M1</sub>, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, *FEBS Letters*, **1987**, 223, 42; Wu *et al.*, *Cancer Research*, **1993**, 53, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al.* (*Ann. N.Y. Acad. Sci.*, **1987**, 507, 64) reported the ability of monosialoganglioside G<sub>M1</sub>, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon *et al.* (*Proc. Natl. Acad. Sci. U.S.A.*, **1988**, 85, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G<sub>M1</sub> or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb *et al.*) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-*sn*-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim *et al.*).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto *et al.* (*Bull. Chem. Soc. Jpn.*, **1980**, 53, 2778) described liposomes comprising a nonionic detergent, 2C<sub>12</sub>15G, that contains a PEG moiety. Illum *et al.* (*FEBS Lett.*, **1984**, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (*e.g.*, PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klivanov *et al.* (*FEBS Lett.*, **1990**, 268, 235) described

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experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume *et al.* (*Biochimica et Biophysica Acta*, 1990, 1029, 91) extended such observations to other PEG-derivatized phospholipids, *e.g.*,  
5 DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.*  
10 (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-modified ceramide lipids are described in WO  
15 96/10391 (Choi *et al.*). U.S. Patent Nos. 5,540,935 (Miyazaki *et al.*) and 5,556,948 (Tagawa *et al.*) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high  
20 molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include RNA. U.S. Patent No. 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et al.* discloses liposomes comprising antisense  
25 oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller  
30 than the droplet. Transfersomes are adaptable to the environment in which they are used, *e.g.* they are self-optimizing (adaptive to the shape of pores in the skin),

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self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated  
5 delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural  
10 and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

15 If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene  
20 glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

25 If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and  
30 sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.



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If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

5        If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

10        The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

#### Penetration Enhancers

15        In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is  
20        treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

25        Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

30        Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial

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tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi *et al.*, *J. Pharm. Pharmacol.*, **1988**, *40*, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C<sub>1-10</sub> alkyl esters thereof (*e.g.*, methyl, isopropyl and *t*-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; El Hariri *et al.*, *J. Pharm. Pharmacol.*, **1992**, *44*, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, **1996**, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium

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taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92;

- 5 Swinyard, Chapter 39 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, **1990**, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33; Yamamoto *et al.*, *J. Pharm. Exp. Ther.*, **1992**, 263, 25; Yamashita *et al.*, *J. Pharm. Sci.*, **1990**, 79, 579-583).

10

- Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as
- 15 penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, **1993**, 618, 315-339). Chelating agents of the invention include but are not limited to disodium
- 20 ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines)(Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33; Buur *et al.*,
- 25 *J. Control Rel.*, **1990**, 14, 43-51).

- Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that
- 30 nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**,

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7, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.*, 1987, 39, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al.*, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

#### Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*,

*Antisense Res. Dev.*, 1995, 5, 115-121; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, 1996, 6, 177-183).

#### Excipients

- 5           In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk,
- 10 consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl
- 15 cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrants (*e.g.*, starch, sodium starch glycolate, *etc.*); and wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*).
- 20           Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium
- 25 stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

- Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil
- 30 bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable

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for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

#### Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more modified oligonucleotide compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin,

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idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. 1987, pp. 1206-1228, Berkow *et al.*, eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow *et al.*, eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more modified oligonucleotide compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional modified oligonucleotide compounds targeted to a second nucleic acid target. Two or more combined compounds may be used together or sequentially.

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The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure  
5 is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can  
10 generally be estimated based on EC<sub>50</sub>s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations  
15 of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.



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**What is Claimed is:**

1. A method of inhibiting the expression of a gene in a biological system expressing said gene, comprising the steps of:

providing a composition comprising a modified siRNA; and

5 contacting said biological system with said modified siRNA under conditions effective to inhibit the expression of said gene;

said modified siRNA comprising at least one RNA strand having at least one modified nucleoside wherein said modified nucleoside has enhanced binding affinity relative to the unmodified RNA nucleoside in the same position.

10

2. The method of claim 1 wherein the sugar moiety of said modified nucleoside has 3'-endo conformational geometry.

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(54) Title: MODIFIED OLIGONUCLEOTIDES FOR USE IN RNA INTERFERENCE

(57) Abstract: The present invention provides modified oligonucleotides for use in the RNA interference pathway of gene modulation. At least one nucleoside has a 2'-modification other than hydroxyl that gives an RNA like 3'-endo sugar conformation. The modified oligonucleotides are also provided having a 5'-phosphate group.



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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/35086

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 48/00; C12Q 1/68

US CL : 514/44; 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Continuation Sheet

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PARRISH, S. et al. Functional Anatomy of a dsRNA Trigger: Differential Requirement for the Two Trigger Strands in RNA Interference. Molecular Cell. November 2000, Vol. 6, pages 1077-1087, especially figures 5 and 6.	1,2
X	ELBASHIR, S. et al. Functional Anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. The EMBO Journal. 2001, Vol. 20, No. 23, pages 6877-6888, see entire document.	1,2

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	"T"
"A" document defining the general state of the art which is not considered to be of particular relevance	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
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**Continuation of B. FIELDS SEARCHED Item 3:**  
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